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Our previous studies suggested that Egr-1 is required for the growth-inhibitory and apoptotic response to ionizing radiation in the prostate cancer cell line PC-3 that lacks wild-type p53 functional protein. The effect of Egr-1 was mediated through the upregulation of TNF-α protein. These results underscore the need to formally study the functional relevance of EGR-1 expression in radiation treated prostate cancer cells. It is hypothesized that radiation induces EGR-1 protein expression in prostatic carcinoma cells leading to the upregulation of TNF-α protein resulting in apoptosis and cell death. To test this hypothesis the following specific aims are proposed: A. Determine the functional and regulatory role of Egr-1 in radiation-inducible apoptosis using prostate cancer cell lines exhibiting a wild-type (LNCaP) and a mutant p53 (DU-145) background. B. Determine the basal and radiation-inducible expression levels of Egr-1 and its target gene TNF-α protein as a function of radiation. Data obtained on Egr-1 and its target genes will be compared to those corresponding to clonogenic survival, growth inhibition and apoptosis profiles. In this way the functional role of Egr-1 in radiation treated prostate cancer cells can be elucidated. To translate these results in a clinical perspective, we will also analyze Egr-1, p53 and TNF-α expression levels and genomic Egr-1 mutations in untreated prostatic tumor specimens.

After the first year of the granting period, the PI obtained NIH funding that had certain overlapping specific aims. Modified specific aims were approved by the Grants Officer and a new statement of work was developed for the remaining granting period. The major significant findings of this proposal are:
(1)   Egr-1 function is necessary for radiation-induced apoptosis.
(2)   The p53 protein is degraded when Egr-1 is absent after radiation exposure.
(3)   Putative Egr-1 binding was identified in the bax promoter.

14. SUBJECT TERMS
Prostate Cancer, Radiation, Apoptosis, EGR-1

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Table of Contents

INTRODUCTION

An analysis of the molecular indicators of cell proliferation and apoptosis may lead to identification and management of prostate cancer patients with inherent resistance to hormone or radiation therapy. Exposure to radiation causes induction of *Egr*-1, which is a zinc finger transcription factor gene. Our previous studies in prostate cancer cells suggested that *Egr*-1 is required for the growth-inhibitory and apoptotic response to ionizing radiation in the prostate cancer cell line PC-3 that lacks wild-type *p53* functional protein. The effect of *Egr*-1 is mediated through the upregulation of TNF-α protein. These results underscore the need to formally study the functional relevance of EGR-1 expression in radiation treated prostate cancer cells. It is hypothesized that radiation induces EGR-1 protein expression in prostatic carcinoma cells leading to the upregulation of TNF-α protein resulting in apoptosis and cell death. To test this hypothesis the following specific aims were proposed: A. Determine the functional and regulatory role of *Egr*-1 in radiation-inducible apoptosis using prostate cancer cell lines exhibiting a wild-type (LNCaP) and a mutant *p53* (DU-145) background. B. Determine the basal and radiation-inducible expression levels of *Egr*-1 and it's target gene TNF-α protein) as a function of radiation dose by immunohistochemistry, Western blot and reverse transcription-polymerase chain reaction. Data obtained on *Egr*-1 and its target genes (basal and radiation-inducible) will be compared to those corresponding to clonogenic survival (analyzed by colony-forming assay), growth inhibition (analyzed by [³H] thymidine incorporation assay) and apoptosis (analyzed by TUNEL and flow cytometry) profiles. To translate these results in a clinical perspective, we will also analyze *Egr*-1, p53 and TNF-α expression levels and genomic *Egr*-1 mutations in untreated prostatic tumor specimens. If this hypothesis is correct, this study will suggest that androgen depletion (hormone) treatment should be combined with radiation therapy to enhance cell-killing response in prostatic tumors exhibiting wild-type functional *Egr*-1 gene.

BODY

In this section, a detailed description of the research accomplishment will be described as per tasks outlined in the approved statement of work. The following tasks pertains to the first year of the granting period:

TASK-1. Radiobiological profile and *Egr*-1 gene characterization in DU-145 and LNCaP cells, months 1- 6:
    a. Radiobiological characteristics of DU-145 and LNCaP cells (colony formation, growth inhibition and apoptosis) and radiation-induced *Egr*-1 protein analysis by Westerns and immunocytochemistry. Months 1-4.
    b. Characterization of *Egr*-1 gene in DU-145 and LNCaP cells (FISH, mutation analysis and sequencing of whole gene). Months 5-6.

TASK-2. Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cells (DU-145 and LNCaP), months 7-18:
    a. Transfections using constructs such as empty vector (pCB6+), the chimera WT1-EGR1 and EGR-1 cDNA will be performed in DU-145 and LNCaP cells. CAT assays will be performed using EBS-CAT construct. Months 7-13.

TASK-4. The clinical relevance of *Egr*-1 mutation and expression in primary tumor specimens of prostate cancer, months 1-30:
    a. Collection of 100 prostate tumor specimens. Months 1-24.
    b. Immunohistochemical and PCR-SSCP analysis of *Egr*-1 gene in prostate tumor specimens. Months 25-29.
    c. Interpretation of results and preparation of manuscript for publication. Month 30.

Task 1 and 2: Radiobiological profile and *Egr*-1 gene characterization in DU-145 and LNCaP cells. Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in DU-145 cells: Transfections using constructs such as empty vector (pCB6+), the chimera WT1-EGR1 and EGR-1 cDNA will be performed in DU-145 cells

Purpose : The present study was undertaken to determine the radiobiological characteristics of prostate cancer cell lines DU-145 and LNCaP and also to determine the structure and functional status of *Egr*-1 gene in these cell lines. This was accomplished by: (1) studying the effect of ionizing radiation on the growth of these cells; (2) analyzing the allelic and mutational status of *Egr*-1 in DU-145 and LNCaP cells; and (3) studying the radio-induction potential of *Egr*-1 protein and also it's target gene TNF-α mRNA DU-145 and LNCaP cells. To directly understand the functional and regulatory role of *Egr*-1 expression in the radiation-inducible signaling pathway in prostate cancer cells, we describe here the effect of *Egr*-1 overexpression or inhibition of *Egr*-1 function on radiation-induced growth inhibition and apoptosis in the DU-145 prostate cancer cell line.

Results: Results pertaining to these two tasks were furnished in the Annual report. The task 1 statement-of-work results relating to the use of LNCaP cells were reported previously. The analysis of aims using the DU-145 cells pertaining to task 1 and task 2 is given in the form of abstract below. Work done on these cells resulted in a manuscript that will be submitted to Journal Biological Chemistry soon. A copy of the manuscript is enclosed herewith in Appendix II along with figures.

EGR-1 overexpression triggers caspase activation and cell death through transcriptional upregulation of Bax in irradiated p53 mutant prostate cancer cell line DU 145 (manuscript enclosed in appendix II): Early growth response-1 gene (*Egr*-1) is a strong transcriptional activator of key genes involved in growth-inhibition and cell death pathway. In this study, we investigated the functional role of EGR-1 in the regulation of radiation-induced clonogenic inhibition and apoptosis in p53 mutant prostate cancer cell line DU145. The clonogenic assays indicated that the DU145/CMV-EGR-1 cells (SF_2 =0.34; D_0=164 cGy) were significantly sensitive to radiation (p<0.0001) and the dominant-negative mutant of EGR-1 transfected cells DU145/CMV-WT1-EGR1, (SF_2=0.66; D_0=509 cGy) were resistant to radiation (p<0.001) when compared to DU145/Vector cells (SF_2=0.609; D_0=400 cGy). TUNEL analysis also showed significant induction of radiation-induced

5

apoptosis in DU145/CMV-EGR-1 transfectant cells, than DU145/Vector cells alone. Diminished induction of radiation-induced apoptosis was evident in DU145/CMV-WT1-EGR1 cells when compared to DU145/Vector cells alone. Enhanced radiation sensitivity of DU145/CMV-EGR-1 cells was associated with up-regulation of Bax at the RNA and protein level. Radiation also caused down regulation of Bcl-2 in DU145/CMV-EGR-1 cells when compared to unaltered levels of Bcl-2 in DU145/Vector and DU145/CMV-WT1-EGR1 cells. In addition, significant activation of Caspase-3 and Caspase-9 with increased PARP cleavage was observed in DU145/CMV-EGR-1 cells when compared to DU145/Vector or DU145/CMV-WT1-EGR1 cells. Gel shift analysis and CAT reporter assay indicated that EGR-1 transactivates the promoter of the Bax gene that contains two overlapping GC-rich EGR-1 binding sites. These findings establish that radiation-induced proapoptotic action of EGR-1, in a mutant p53 background, directly transactivates Bax that alters the Bcl-2: Bax ratio resulting in significant activation of caspases and induction of cell death pathway.

Task 4: **The clinical relevance of *Egr*-1 mutation and expression in primary tumor specimens of prostate cancer**

Purpose: This study was undertaken to determine whether EGR-1 overexpression in the primary tumor correlates with radiation response either in terms of complete local control with no evidence of disease or recurrence / evidence of metastasis.

Results: The results of this task was submitted to Journal of Clinical Oncology and this is accepted for publication. This will be published in the month of August 2001. A manuscript is enclosed in Appendix II. The abstract of this manuscript is given below:

EARLY GROWTH RESPONSE-1 GENE: A POTENTIAL RADIATION RESPONSE GENE MARKER IN PROSTATE CANCER (manuscript enclosed in appendix II): This study was undertaken to determine whether the transcription factor EGR-1 expression (1) in the primary tumor correlates with radiation response in terms of complete local tumor control with no evidence of disease or recurrence / evidence of metastasis; (2) in the post-irradiated biopsies correlates with residual tumor; and (3) correlates with the expression of *Egr*-1 target genes such as p53, pRB and Bax. We analyzed (a) 25 pretreated surgically resected paraffin-embedded primary adenocarcinomas of the prostate for the presence of the EGR-1 expression and mutation and correlated with clinical end-points such as serum PSA levels and current clinical status; (b) 27 post-irradiated biopsies of prostate for the presence of EGR-1 expression and to correlate these findings to the residual tumor status; and (c) 12 prospective prostate tumor specimens for EGR-1 expression and its target genes. EGR-1 expression was determined by immunohistochemistry and mutations were screened in two regions of *Egr*-1 gene (trinucleotide AGC repeats in transactivation domain (TD) and poly A tract in 3'UTR) by PCR-SSCP analysis. Out of 25 patients, 18 patients showed expression of EGR-1. Nuclear staining was weak or usually absent in non-malignant regions. Poorly differentiated carcinoma showed more intense nuclear staining than moderately differentiated tumors. Out of 18 patients with EGR-1 expression, five had mutation in the transactivation domain and one had mutation in both transactivation and 3'UTR region. EGR-1 overexpression correlated with treatment failure. Additionally, in post-irradiated biopsy specimens, 8 of 9 cases with residual tumor (18 out of 27 were benign) showed intense EGR-1 staining only in the carcinoma. No correlation with EGR-1 overexpression and its' target genes were found which may indirectly suggest that overexpressed EGR-1 may lack transactivation function. In summary, EGR-1 overexpression in the mutant form may provide an indication of clinical failure (local recurrence or metastasis).

Change in the statement of work

Based on the work supported by this award, Principal Investigator received RO-1 funding from National Cancer Institute, NIH starting from August 1, 1999. Since there was a significant overlap between the remaining Statement of Work of this award and the new grant, the Grants Officer of USAMRMC approved new Statement of Work for the remaining granting period (Revised Assistance Agreement is enclosed in Appendix II). The new Statement of work for the period August 1, 1999 to April 30, 2001 is shown below:

TASK-1. Radiobiological profile and *Egr*-1 gene characterization in CWR22R cells, months 1- 4:

a. Radiobiological characteristics of CWR22R and CWR22 cells (colony formation, growth inhibition and apoptosis) and radiation-induced *Egr*-1 protein analysis by Westerns and immunocytochemistry. Months 1-3.

b. Characterization of *Egr*-1 gene in CWR22R and CWR22 cells (FISH, mutation analysis and sequencing of whole gene). Month 4.

TASK-2. Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22), months 9-14:

a. Transfections using constructs such as empty vector (pCB6+), the chimera WT1-EGR1 and EGR-1 cDNA will be performed in CWR22R and CWR22 cells. CAT assays will be performed using EBS-CAT construct. Months 9-11.

b. Radiobiological characteristics of stable transfectant (CWR22R and CWR22) cells (colony formation, growth inhibition and apoptosis). Months 12-13.

c. Interpretation of results and preparation of manuscript for publication. Month 14.

TASK-3. Mechanism of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22) by *Egr*-1 mediated up-regulation of the TNF-α gene, months 15-18:

a. CAT assays using p53 (2.2+1.6)-CAT, pRB-CAT and TNFp-CAT constructs in parental and stably transfected CWR22R and CWR22 cells. Gel-shift assays in parental and stably transfected CWR22R and CWR22 cells. Months 15-17.

b. Interpretation of results and preparation of manuscript for publication. Month 18.

TASK-4. To understand the mechanism of radiation-induced apoptosis in isogenic mouse embryonic fibroblast (MEF) cells with homozygous (Egr-1$^{+/+}$), heterozygous (Egr-1$^{+/-}$) and null (Egr-1$^{-/-}$) allelic status for Egr-1 gene. Months 5-8.

a. Western blot and RT-PCR analysis of p53, TNF-α and pRB genes in untreated and irradiated MEFs with Egr-1+/+ and Egr-1-/- background. Month 5.

b. CAT assays for p53, TNF-α and pRB. Month 6.

c. Immunoblot analysis of p53, mdm-2 and pRB. Month 7

Task 1, 2 and 3:   **Radiobiological profile and *Egr*-1 gene characterization in CWR22 and CWR22R cells. Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22). Mechanism of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22) by *Egr*-1 mediated up-regulation of the TNF-α gene. *(All figures pertaining to this task are enclosed in Appendix I)***

Purpose: Induction of *Egr-1* by radiation is expected to activate transcription via the GC-rich DNA binding sites present in several promoters of downstream genes leading to alteration in their expression profile and a phenotypic response. A recent report suggests that the *Egr-1* gene is one of a growing set of tumor suppressor-type genes (1). In order to understand the role of *Egr-1* in growth control of prostate cancer cells, this project proposes to investigate the functional relevance of radiation-inducible transient induction of EGR-1 protein in clonogenic inhibition or cell death. Until date, there are 9 human prostate cancer cell lines and 21 xenograft tumors, derived from primary and metastatic human prostate tumors (2-4). Two xenograft tumors (CWR22 and CWR22R) will be used in this specific aim. CWR22 was derived from Gleason tumor grade 9, stage D prostate carcinoma with osseous metastasis. It is androgen-dependent, does not grow in female mice, and regresses in male mice after orchiectomy. CWR22R, a subline of CWR22 that recurred after regression upon androgen withdrawal, is not dependent on androgen and is able to grow in female and castrated animals. Thus, CWR22 and CWR22R represent the first pair of human androgen-dependent and –independent xenograft tumors derived from the same patient tumor. These two xenograft cell lines will form excellent model to study the *Egr*-1 mediated regulation of radiation-inducible apoptosis in paired androgen-dependent and –independent prostate tumors.

Results: **Radiobiological profile and *Egr*-1 gene characterization in CWR22R cells:** CWR22 and CWR22R cells which are the first pair of human androgen-dependent and –independent xenograft tumors respectively, derived from the same patient tumor (J. Natl. Cancer Inst. 85:396, 1993). Clonogenic assays indicated that the CWR22R cells (SF$_2$=0.55; D$_0$=276 cGy) were resistant to radiation (Figure 1). CWR22 cells

7

failed to form colonies after three repeated experimental attempts. TUNEL assays indicated that androgen dependent cells (CWR22) were sensitive to radiation than androgen-independent cells (CWR22R) (Figure 2). Both the cell lines have EGR-1 alleles. Radiation caused a modest increase in EGR-1 protein in CWR22R cells (Figure 3a). The transactivation potential of endogenous was low in response to ionization radiation in CWR22R cells. Sequence analysis of Egr-1 in both cell lines indicated no presence of mutation. The CWR22R cells had two intact Egr-1 alleles as demonstrated by FISH analysis.

Failure to maintain the cultures of androgen dependent CWR22 cells and change of cell line for this study: Except for TUNEL and sequencing experiments, rest of the analyses were not be able to performed in the androgen dependent CWR22 cells because of problems encountered in maintaining the cultures. When consulted with Dr Pretlow, the originator of this cell line, it was suggested to passage these cells in animals in presence of testosterone. Because of the time limitation in terms applying for IACUC approval and DEA (Drug Enforcement Agency) approval to get testosterone pellets, we decided to substitute this cell line with HPV-transformed normal prostate epithelial cell PZ-HPV7. Our previous data has demonstrated clearly that EGR-1 function is necessary for radiation-induced apoptosis. In addition, overexpression of EGR-1 protein in prostate cancer cell lines such as PC-3 and DU-145 led to radiation sensitization. Thus, the use normal prostate cell line PZ-HPV7 to study the influence of EGR-1 overexpression on radiosensitization potential is important in long term for gene therapy strategies. Clonogenic assays indicated that the PZ-HPV7 cells (SF_2=0.49; D_0=206 cGy) were resistant to radiation (Figure 1) and these cells were slightly radiosensitive when compared to androgen-independent CWR22R cells. TUNEL analysis showed that both the cell lines were resistant to radiation-induced apoptosis (Figure 2). Radiation caused a modest increase in EGR-1 gene (Figure 3b). PZ-HPV-7 has two intact alleles for Egr-1 with no obvious mutation in the cDNA sequence.

Overexpression of EGR-1 protein in androgen-independent cell line, CWR22R, led to significant sensitization to ionizing radiation inducible clonogenic inhibition and apoptosis: CWR22R cells were infected with Ad/GFP or Ad/GFP-EGR-1 or Ad/GFP-NAB-1 (natural co-repressor of Egr-1) and irradiated to assess the impact on clonogenic inhibition and apoptosis. The impact on EGR-1 expression in CWR22R cells at various multiplicity of infection (MOI) is shown in Figure 4. The clonogenic assays indicated that the CWR22R/Ad-EGR-1 cells (SF_2 =0.29; D_0=151 cGy) were significantly sensitive to radiation (p<0.0001) and the co-repressor of EGR-1 infected cells CWR22R/Ad-NAB-1, (SF_2=0.63; D_0=340 cGy) were resistant to radiation (p<0.001) when compared to CWR22R/Ad-GFP cells (SF_2=0.45; D_0=270 cGy) (Figure 5). TUNEL analysis also showed significant induction of radiation-induced apoptosis in CWR22R/Ad-EGR-1 transfectant cells, than CWR22R/Ad-GFP cells alone. However, no significant difference in the induction of radiation-induced apoptosis was evident in CWR22R/Ad-NAB-1cells when compared to CWR22R/Ad-GFP cells alone (Figure 6). Enhanced radiation sensitivity of CWR22R/Ad-EGR-1 cells was associated with significant induction of Bax protein (Figure 7).

Overexpression of EGR-1 protein in HPV transformed normal prostate cell line, PZ-HPV-7, led to marginal sensitization to ionizing radiation inducible clonogenic inhibition: PZ-HPV-7 cells were infected with Ad/GFP or Ad/GFP-EGR-1 or Ad/GFP-NAB-1 (natural co-repressor of Egr-1) and irradiated to assess the impact on clonogenic inhibition and apoptosis. The clonogenic assays indicated that the PZ-HPV-7 /Ad-EGR-1 cells (SF_2 =0.37; D_0=169 cGy) showed marginal sensitivity to radiation and the co-repressor of EGR-1 infected cells PZ-HPV-7 /Ad-NAB-1, (SF_2=0.44; D_0=264 cGy) were resistant to radiation when compared to PZ-HPV-7 /Ad-GFP cells (SF_2=0.42; D_0=213 cGy) (Figure 8). However, TUNEL analysis showed significant induction of radiation-induced apoptosis in PZ-HPV-7 /Ad-EGR-1 transfectant cells, than PZ-HPV-7 /Ad-GFP cells alone. And, no significant difference in the induction of radiation-induced apoptosis was evident in PZ-HPV-7 /Ad-NAB-1cells when compared to PZ-HPV-7 /Ad-GFP cells alone (Figure 9). The marginal induction of radiation sensitivity in PZ-HPV-7 /Ad-EGR-1 cells was associated with significant induction of Bax protein (Figure 10).

Inference from these tasks: Studies pertaining to Egr-1 and its' target gene were performed using gel-shift assays, CAT-reporter assays and RT-PCR analysis for p53 and TNF-α. The results of these experiments showed that Egr-1 is functional in both the parental cell lines. However, overexpression of EGR-1 protein resulted in significant induction of Bax protein in both the cell lines. The levels of Bax protein in both cell lines was not detected in parental cells. Induction of Bax was associated with enhanced clonogenic inhibition and apoptosis in CWR22R. However, in PZ-HPV-7 cells, marginal sensitization to radiation-induced clonogenic

inhibition and enhanced sensitization to radiation-induced apoptosis was observed. Altogether, these results imply that overexpression of EGR-1 causes enhance sensitization to radiation-induced killing in prostate tumor cells. However, the effect of EGR-1 on normal prostate cells needs to be examined further due differential sensitization in terms of clonogenic inhibition and apoptosis. *(All figures pertaining to this task are enclosed in Appendix I)*

Task 4: **To understand the mechanism of radiation-induced apoptosis in isogenic mouse embryonic fibroblast (MEF) cells with homozygous (Egr-1$^{+/+}$), heterozygous (Egr-1$^{+/-}$) and null (Egr-1$^{-/-}$) allelic status for Egr-1 gene.**

Purpose: In our previous studies, we used tumor cell lines such as wild-type p53 melanoma cells (5) and p53-deficient prostate cancer cells (6) to understand the regulatory role of *Egr*-1 in apoptotic processes. These studies strongly suggested that *Egr*-1 can mediate its apoptotic action irrespective of *p53* status. However, in a recent report, it was found that *Egr*-1 transactivates the promoter of *p53* gene and up-regulates *p53* mRNA and protein levels in response to apoptotic stimuli (7). This prompted us to further investigate the interactive role of *Egr*-1 with *p53* during the process of apoptosis. We sought to investigate this mechanism in normal cell background with varied genomic status for *Egr*-1 gene (cells with both intact *Egr*-1 alleles, homozygous and heterozygous deletion for *Egr*-1 gene). Mouse embryonic fibroblast (MEF) cells established from homozygous (*Egr*-1$^{-/-}$) and heterozygous (*Egr*-1$^{+/-}$) *Egr*-1 knock-out mice were kindly provided by Dr Jeffrey Milbrandt, Dept of Pathology, Washington University, St.Louis (8). MEFs from normal mice containing intact alleles for *Egr*-1 were established in the laboratory. The results of the study was published in Journal of Biological Chemistry (9) and this publication in enclosed in Appendix II. The abstract is given below:

IONIZING RADIATION DOWN-REGULATES P53 PROTEIN IN PRIMARY *EGR*-1$^{-/-}$ MOUSE EMBRYONIC FIBROBLAST CELLS CAUSING ENHANCED RESISTANCE TO APOPTOSIS (publication reprint is enclosed in appendix II): In this study, we sought to investigate the mechanism of the proapoptotic function of *Egr*-1 in relation to p53 status in normal isogenic cell backgrounds by using primary MEF cells established from homozygous (*Egr*-1$^{-/-}$) and heterozygous (*Egr*-1$^{+/-}$) *Egr*-1 knockout mice. Ionizing radiation caused significantly enhanced apoptosis in *Egr*-1$^{+/-}$ cells (22.8%; p<0.0001) when compared to *Egr*-1$^{-/-}$ cells (3.5%). Radiation elevated p53 protein in *Egr*-1$^{+/-}$ cells in 3 to 6 hours. However, in *Egr*-1$^{-/-}$ cells, the p53 protein was downregulated in 1 hour after radiation and was completely degraded at the later time points. Radiation elevated the p53-CAT activity in *Egr*-1$^{+/-}$ cells, but not in *Egr*-1$^{-/-}$ cells. Interestingly, transient overexpression of EGR-1 in p53$^{-/-}$ MEF cells caused marginal induction of radiation-induced apoptosis when compared to p53$^{+/+}$ MEF cells. Together, these results indicate that *Egr*-1 may transregulate p53 and both EGR-1 and p53 functions are essential to mediate radiation induced apoptosis. RB, an *Egr*-1 target gene, forms a trimeric complex with p53 and MDM2 to prevent MDM2 mediated p53 degradation. Low levels of RB including hypophosphorylated forms were observed in *Egr-1*$^{-/-}$ MEF cells before and after radiation when compared to the levels observed in *Egr-1*$^{+/-}$ cells. Elevated amounts of the p53-MDM2 complex and low amounts of RB-MDM-2 complex were observed in *Egr-1*$^{-/-}$ cells after radiation. Because of a reduction in RB binding to MDM2 and an increase in MDM2 binding with p53, p53 is directly degraded by MDM2 and this leads to inactivation of the p53-mediated apoptotic pathway in *Egr-1*$^{-/-}$ MEF cells. Thus, the proapoptotic function of *Egr*-1 may involve the mediation of RB protein that is essential to overcome the antiapoptotic function of MDM2 on p53.

KEY RESEARCH ACCOMPLISHMENTS

1. Findings in DU-145 cells establish that radiation-induced proapoptotic action of EGR-1, in a mutant p53 background, directly transactivates Bax that alters the Bcl-2: Bax ratio resulting in significant activation of caspases and induction of cell death pathway. Thus, identification of putative Egr-1 binding site on the Bax promoter is an important key accomplishment.

2. EGR-1 overexpression in the mutant form may provide an indication of clinical failure (local recurrence or metastasis).

3. Overexpression of EGR-1 causes enhance sensitization to radiation-induced killing in prostate tumor cells. However, the effect of EGR-1 on normal prostate cells needs to be examined further due differential sensitization in terms of clonogenic inhibition and apoptosis.

4. Studies using Egr-1 knock-out MEF cells strongly suggest that Egr-1 function is necessary for radiation-induced apoptosis.

5. The proapoptotic function of *Egr*-1 may involve the mediation of RB protein that is essential to overcome the antiapoptotic function of MDM2 on p53.

REPORTABLE OUTCOMES

The following presentations were made in the National Meetings:

1. Ahmed, M.M., Chendil, D., Dey, S., Parekh, S., Mohiuddin, M., Rangnekar, V.M. and Milbrandt, J.D. Ionizing radiation down-regulates *p53* protein in *Egr*-1⁻/⁻ MEF cells causing enhanced resistance to apoptosis. Presented at 90th Annual Conference of American Society for Cancer Research (1999) **Philadelphia, PA.**

2. Ahmed, M.M., Chendil, D., Dey, S., Parekh, S., Mohiuddin, M., Rangnekar, V.M. and Milbrandt, J.D. Ionizing radiation down-regulates *p53* protein in *Egr*-1⁻/⁻ MEF cells causing enhanced resistance to apoptosis. Presented at Gordon Research Conference on "Radiation Oncology" (1999) at **Ventura, CA.**

3. Das, A., Chendil, D., Dey, S., Parekh, S., Mohiuddin, M., Rangnekar, V.M and **Ahmed, M.M.** Degradation of *p53* protein in irradiated *Egr*-1⁻/⁻ MEF cells led to enhanced radioresistance. Presented at 47th Annual Meeting of the Radiation Research Society (April-May, 2000) **Albuquerque, NM (RADIATION RESEARCH SOCIETY YOUNG SCIENTIST AWARD).**

4. Das, A., Chendil, D., Dey, S., Mohiuddin, M., Rangnekar, V.M. and **Ahmed, M.M.** Overexpression of EGR-1 in p53 mutant prostate cancer cell line DU-145 enhances radiosensitivity by altering bcl-2:bax ratio. Presented at 91st Annual Conference of American Association for Cancer Research (April, 2000) **San Francisco, CA. (ORAL PRESENTATION).**

Publications

1. Das, A., Chendil, D., Dey, S., Mohiuddin, M., Mohiuddin, M., Milbrandt, J., Rangnekar, V.M and **Ahmed, M.M.** Ionizing radiation down-regulates p53 protein in primary Egr-1⁻/⁻ mouse embryonic fibroblast cells causing enhanced resistance to apoptosis. **Journal of Biochemistry** 276 (5):3279-3286, 2001.

2. Ahmed, M.M., Chendil, D., Lele, S., Venkatasubbarao, K., Dey, S., Ritter, M., Rowland, R.G. and Mohiuddin, M. Early Growth Response-1 gene: a potential radiation response gene marker in prostate cancer. Journal of Clinical Oncology (In press).

Manuscript under preparation:

Das, A., Chendil, D., Dey, S., Mohiuddin, M. and Ahmed, M.M. EGR-1 overexpression triggers caspase activation and cell death through transcriptional upregulation of Bax in irradiated p53 mutant prostate cancer cell line DU 145

Funding based on work supported by this award:

Base on the work supported by this award, Principal Investigator received RO-1 funding from National Cancer Institute, NIH starting from August 1, 1999 (Critique and Award letter is enclosed in Appendix II). Since there was a significant overlap between the remaining Statement of Work of this award and this new grant, the Grants Officer of USAMRMC approved new Statement of Work for the remaining granting period (Revised Assistance Agreement is enclosed in Appendix II). The new Statement of work beginning from August 1, 1999 is shown below:

TASK-1. Radiobiological profile and *Egr*-1 gene characterization in CWR22R cells, months 1- 4:

    a.   Radiobiological characteristics of CWR22R and CWR22 cells (colony formation, growth inhibition and apoptosis) and radiation-induced *Egr*-1 protein analysis by Westerns and immunocytochemistry. Months 1-3.

    b.   Characterization of *Egr*-1 gene in CWR22R and CWR22 cells (FISH, mutation analysis and sequencing of whole gene). Month 4.

TASK-2. Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22), months 9-14:

a. Transfections using constructs such as empty vector (pCB6+), the chimera WT1-EGR1 and EGR-1 cDNA will be performed in CWR22R and CWR22 cells. CAT assays will be performed using EBS-CAT construct. Months 9-11.

b. Radiobiological characteristics of stable transfectant (CWR22R and CWR22) cells (colony formation, growth inhibition and apoptosis). Months 12-13.

c. Interpretation of results and preparation of manuscript for publication. Month 14.

TASK-3. Mechanism of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22) by *Egr*-1 mediated up-regulation of the TNF-a gene, months 15-18:

a. CAT assays using p53 (2.2+1.6)-CAT, pRB-CAT and TNFp-CAT constructs in parental and stably transfected CWR22R and CWR22 cells. Gel-shift assays in parental and stably transfected CWR22R and CWR22 cells. Months 15-17.

b. Interpretation of results and preparation of manuscript for publication. Month 18.

TASK-4. To understand the mechanism of radiation-induced apoptosis in isogenic mouse embryonic fibroblast (MEF) cells with homozygous (Egr-$1^{+/+}$), heterozygous (Egr-$1^{+/-}$) and null (Egr-$1^{-/-}$) allelic status for Egr-1 gene. Months 5-8.

a. Western blot and RT-PCR analysis of p53, TNF-a and pRB genes in untreated and irradiated MEFs with Egr-1+/+ and Egr-1-/- background. Month 5.

d. CAT assays for p53, TNF-α and pRB. Month 6.

e. Immunblot analysis of p53, mdm-2 and pRB. Month 7

c. Interpretation of results and preparation of manuscript for publication. Month 8.

CONCLUSIONS

Studies using genetically matched *Egr*-1 MEF cells confirmed that *Egr*-1 is the upstream regulator of apoptosis. The role of *p53* is pivotal in *Egr*-1 mediated apoptosis. The findings of these results indicate that *Egr*-1 may transregulate p53 and both EGR-1 and p53 functions are essential to mediate radiation induced apoptosis. RB, an *Egr*-1 target gene, forms a trimeric complex with p53 and MDM2 to prevent MDM2 mediated p53 degradation. Low levels of RB including hypophosphorylated forms were observed in *Egr-1*$^{-/-}$ MEF cells before and after radiation when compared to the levels observed in *Egr-1*$^{+/-}$ cells. Elevated amounts of the p53-MDM2 complex and low amounts of RB-MDM-2 complex were observed in *Egr-1*$^{-/-}$ cells after radiation. Because of a reduction in RB binding to MDM2 and an increase in MDM2 binding with p53, p53 is directly degraded by MDM2 and this leads to inactivation of the p53-mediated apoptotic pathway in *Egr-1*$^{-/-}$ MEF cells. Thus, the proapoptotic function of *Egr*-1 may involve the mediation of RB protein that is essential to overcome the antiapoptotic function of MDM2 on p53.

Enhanced radiation sensitivity of DU145/CMV-EGR-1 cells was associated with up-regulation of Bax at the RNA and protein level. Radiation also caused down regulation of Bcl-2 in DU145/CMV-EGR-1 cells when compared to unaltered levels of Bcl-2 in DU145/Vector and DU145/CMV-WT1-EGR1 cells. In addition, significant activation of Caspase-3 and Caspase-9 with increased PARP cleavage was observed in DU145/CMV-EGR-1 cells when compared to DU145/Vector or DU145/CMV-WT1-EGR1 cells. Gel shift analysis and CAT reporter assay indicated that EGR-1 transactivates the promoter of the Bax gene that contains two overlapping GC-rich EGR-1 binding sites. These findings establish that radiation-induced proapoptotic action of EGR-1, in a mutant p53 background, directly transactivates Bax that alters the Bcl-2: Bax ratio resulting in significant activation of caspases and induction of cell death pathway.

Studies pertaining to Egr-1 and its' target gene were performed using gel-shift assays, CAT-reporter assays and RT-PCR analysis for p53 and TNF-α. The results of these experiments showed that Egr-1 is functional in both the parental androgen-independent prostate cancer cell line, CWR22R and HPV transformed normal prostate cell lines. However, overexpression of EGR-1 protein resulted in significant induction of Bax protein in both the cell line, PZ-HPV-7. The levels of Bax protein in both cell lines was not detected in parental cells. Induction of Bax was associated with enhanced clonogenic inhibition and apoptosis in CWR22R. However, in PZ-HPV-7 cells, marginal sensitization to radiation-induced clonogenic inhibition and enhanced sensitization to radiation-induced apoptosis was observed. Altogether, these results imply that overexpression of EGR-1 causes enhance sensitization to radiation-induced killing in prostate tumor cells. However, the effect of EGR-1 on normal prostate cells needs to be examined further due differential sensitization in terms of clonogenic inhibition and apoptosis.

This study indicated that the incidence of EGR-1 overexpression is high in the prostate adenocarcinoma. Out of 25 patients, 18 patients showed expression of EGR-1. Nuclear staining was weak or usually absent in non-malignant regions. Poorly differentiated carcinoma showed more intense nuclear staining than moderately differentiated tumors. Out of 18 patients with EGR-1 expression, five had mutation in the transactivation domain and one had mutation in both transactivation and 3'UTR region. EGR-1 overexpression correlated with treatment failure. Additionally, in post-irradiated biopsy specimens, 8 of 9 cases with residual tumor (18 out of 27 were benign) showed intense EGR-1 staining only in the carcinoma. No correlation with EGR-1 overexpression and its' target genes were found which may indirectly suggest that overexpressed EGR-1 may lack transactivation function. In summary, EGR-1 overexpression in the mutant form may provide an indication of clinical failure (local recurrence or metastasis).

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Figure 1. Radiation-induced clonogenic inhibition in CWR22R and PZ-HPV-7 cells.
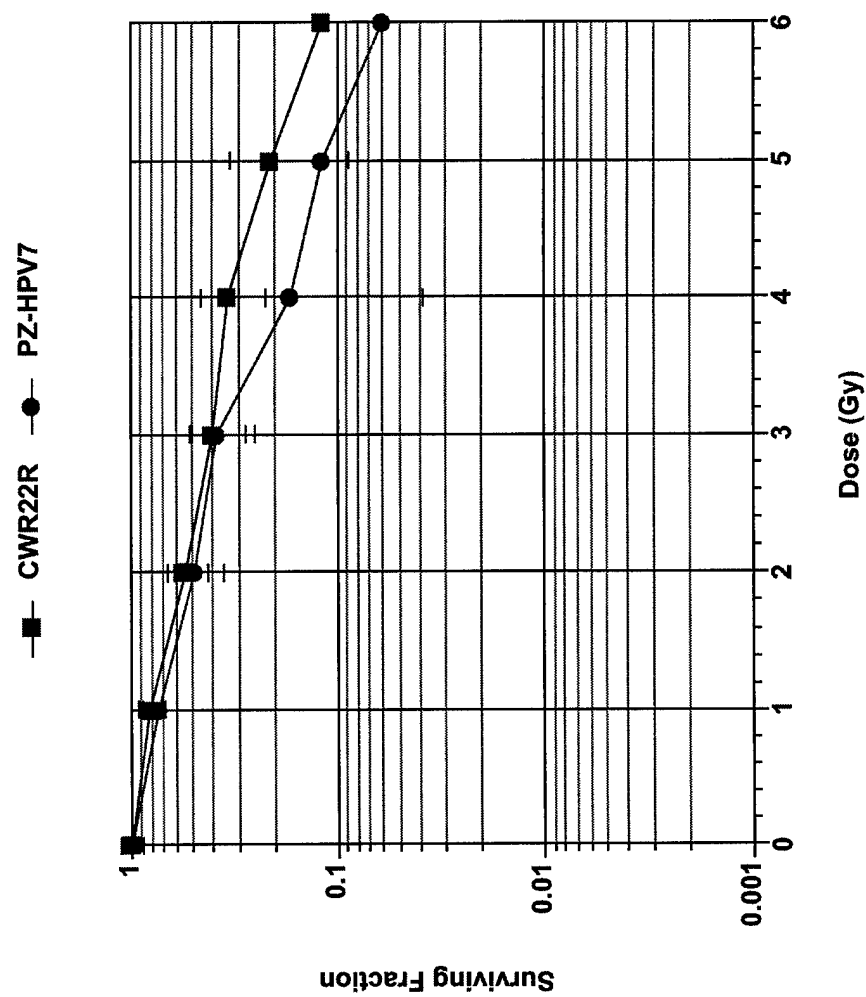
Figure 2. Radiation-induced apoptosis in CWR22R and PZ-HPV-7 cells. The cells were left untreated or irradiated at 5 Gy and TUNEL assay was performed. The untreated levels were normalized with the irradiated groups.
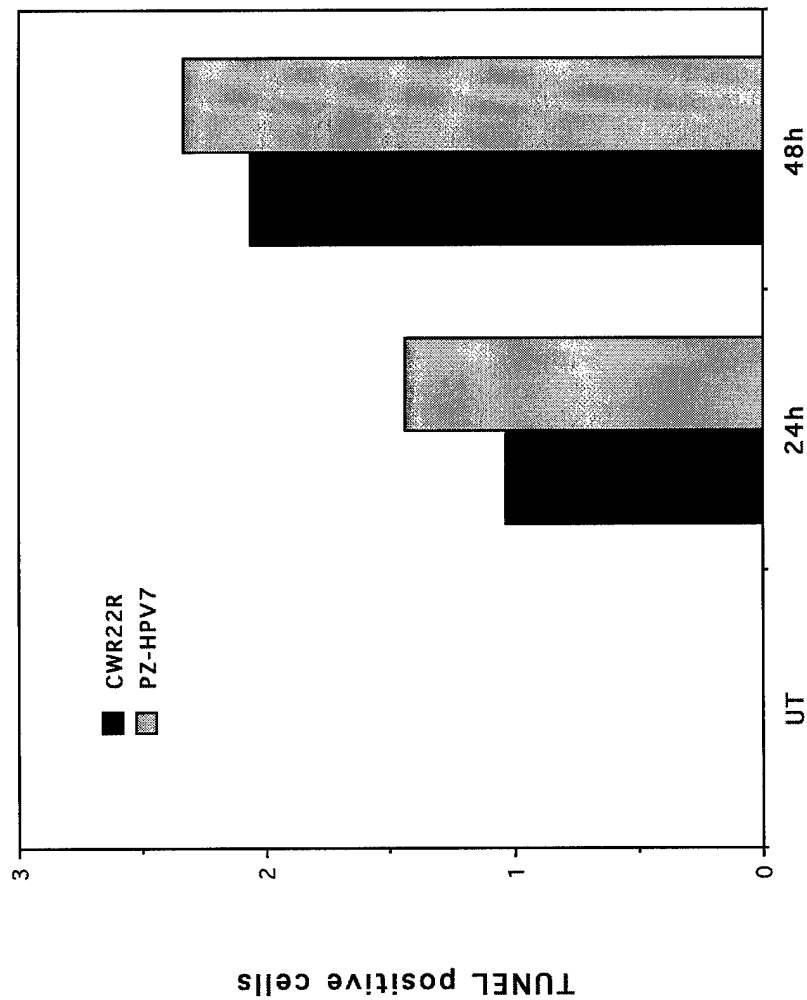
Figure 3A. Western blot analysis of gene expression protein kinetics of CWR22R, androgen independent prostate cancer cell line. Whole cell protein extracts were prepared from CWR22R cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for time interval indicated. The blot was probed with an antibody for EGR-1, p53, Bcl-2, pRB or β–actin.

Figure 3B. Western blot analysis of gene expression protein kinetics of PZ-HPV-7 HPV-transformed normal prostate cells. Whole cell protein extracts were prepared from PZ-HPV-7 cells that were left untreated (UT) or exposed to a 5 Gy dose and incubated for time interval indicated. The blot was probed with an antibody for EGR-1, p53, Bcl-2 or β–actin.
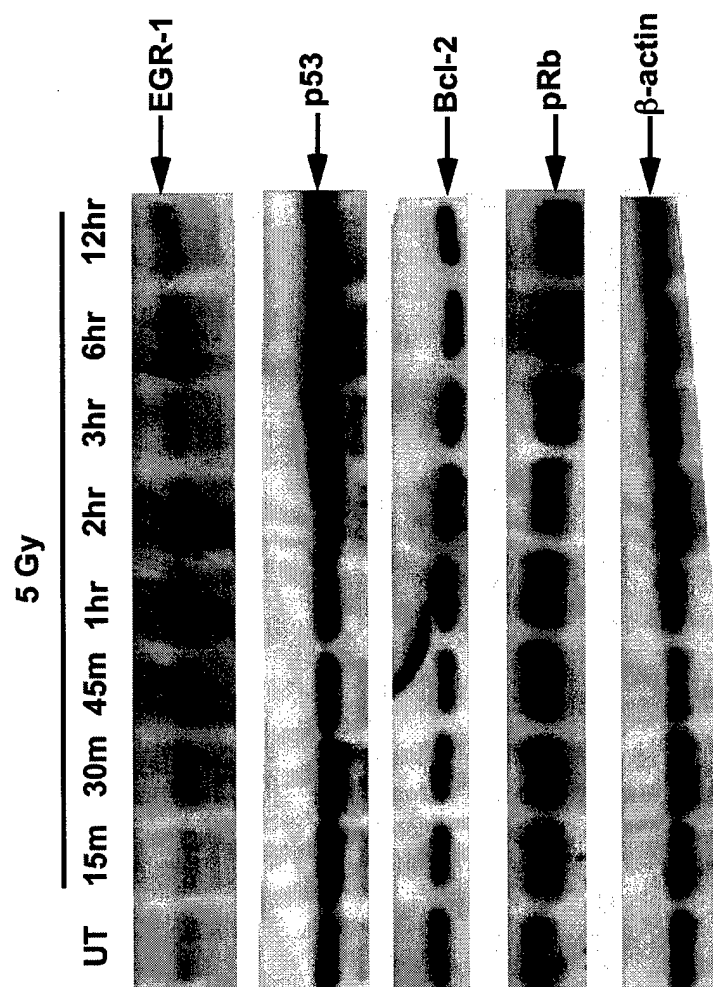
Figure 4. Adenoviral infection of GFP, EGR-1 and NAB-1 in CWR22R cells. Whole protein cell lysates were prepared after 48 hours after infection and Western blot analysis was performed for EGR-1 and β-actin.
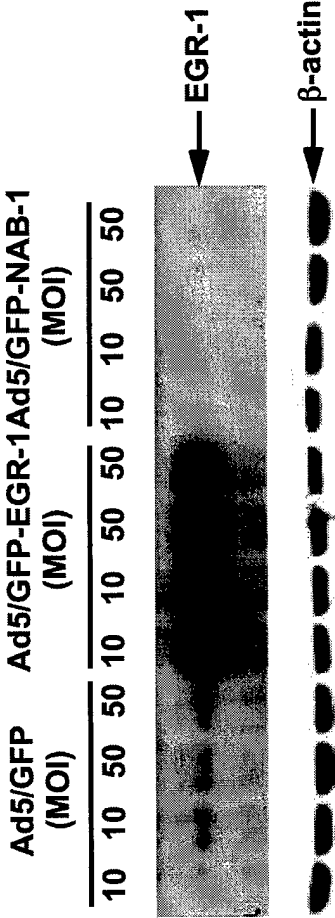
Figure 5. Radiation-induced clonogenic inhibition in CWR22R cells infected with adenoviral vectors containing GFP or EGR-1 or NAB1
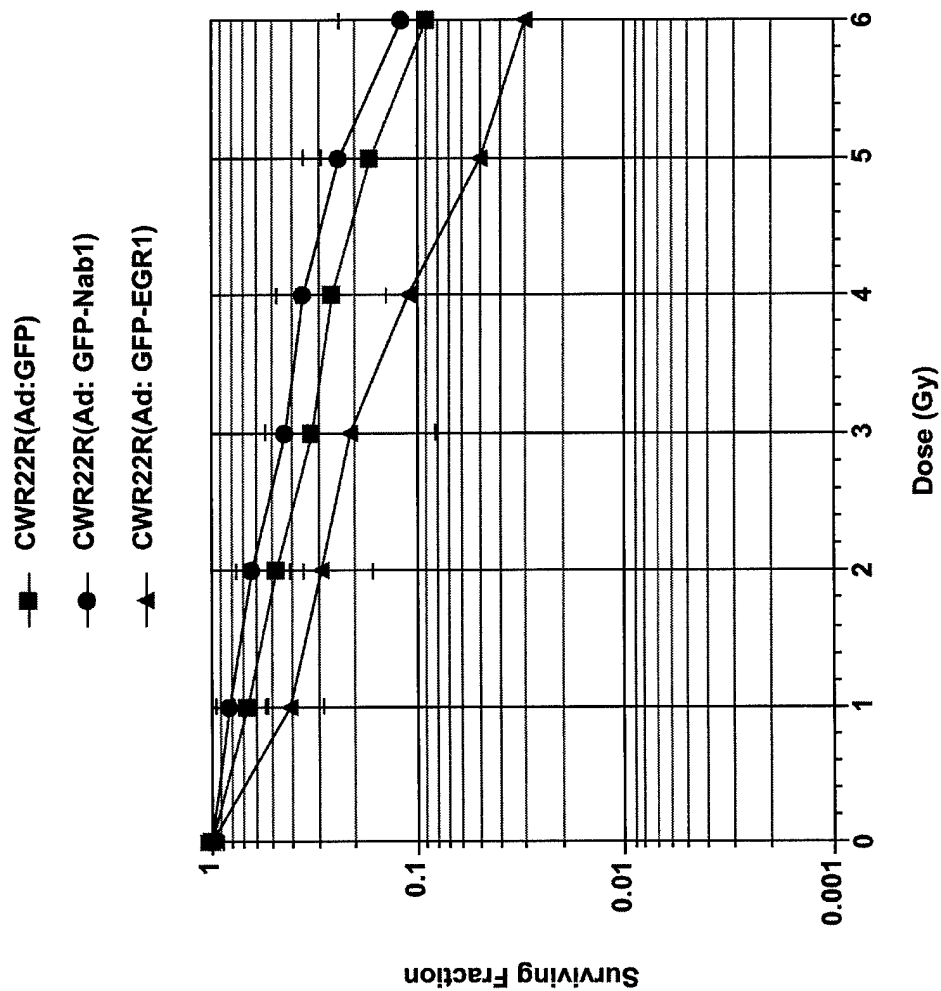
Figure 6. Radiation-induced apoptosis in CWR22R infectant cells. The cells were left untreated or irradiated at 5 Gy and TUNEL assay was performed. The untreated levels were normalized with the irradiated groups.
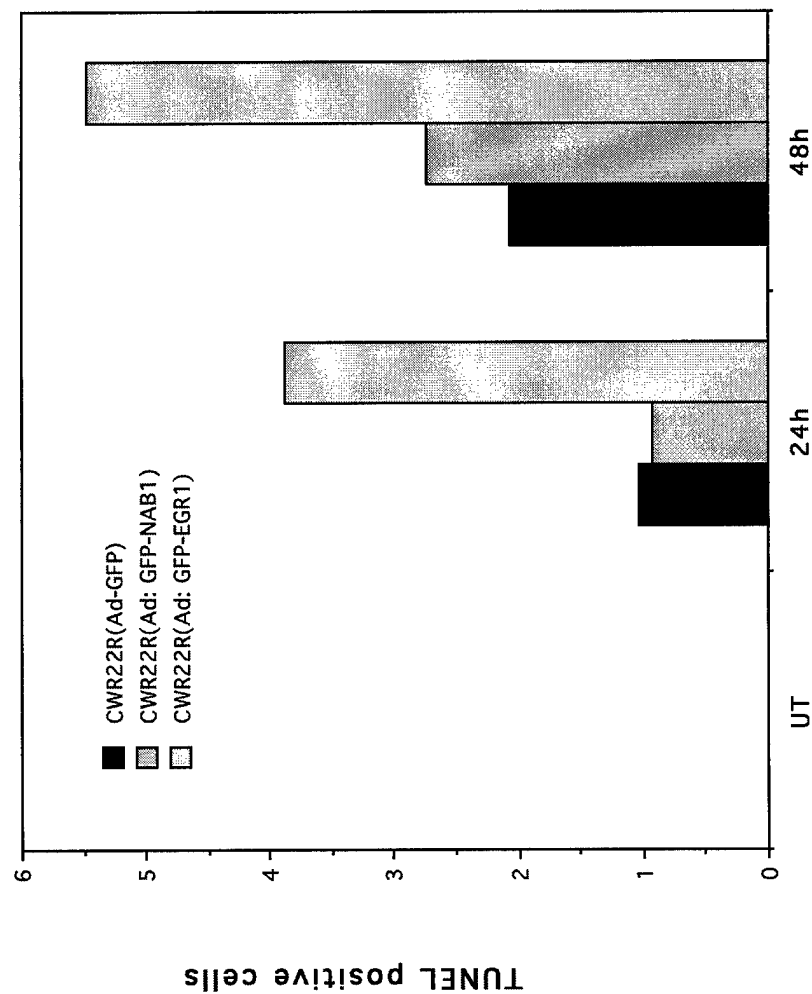
Figure 7A. Western blot analysis of gene expression protein kinetics of CWR22R cells infected with Ad/GFP. Whole cell protein extracts were prepared from these infectant cells and were left untreated (UT) or exposed to a 5 Gy dose and incubated for time interval indicated. The blot was probed with an antibody for EGR-1, p53, Bcl-2, Bax or β–actin.

Figure 7B. Western blot analysis of gene expression protein kinetics of CWR22R cells infected with Ad/GFP-EGR-1. Whole cell protein extracts were prepared from these infectant cells and were left untreated (UT) or exposed to a 5 Gy dose and incubated for time interval indicated. The blot was probed with an antibody for EGR-1, p53, Bcl-2, Bax or β–actin.

Figure 7C. Western blot analysis of gene expression protein kinetics of CWR22R cells infected with Ad/GFP-NAB-1. Whole cell protein extracts were prepared from these infectant cells and were left untreated (UT) or exposed to a 5 Gy dose and incubated for time interval indicated. The blot was probed with an antibody for EGR-1, p53, Bcl-2, Bax or β–actin.
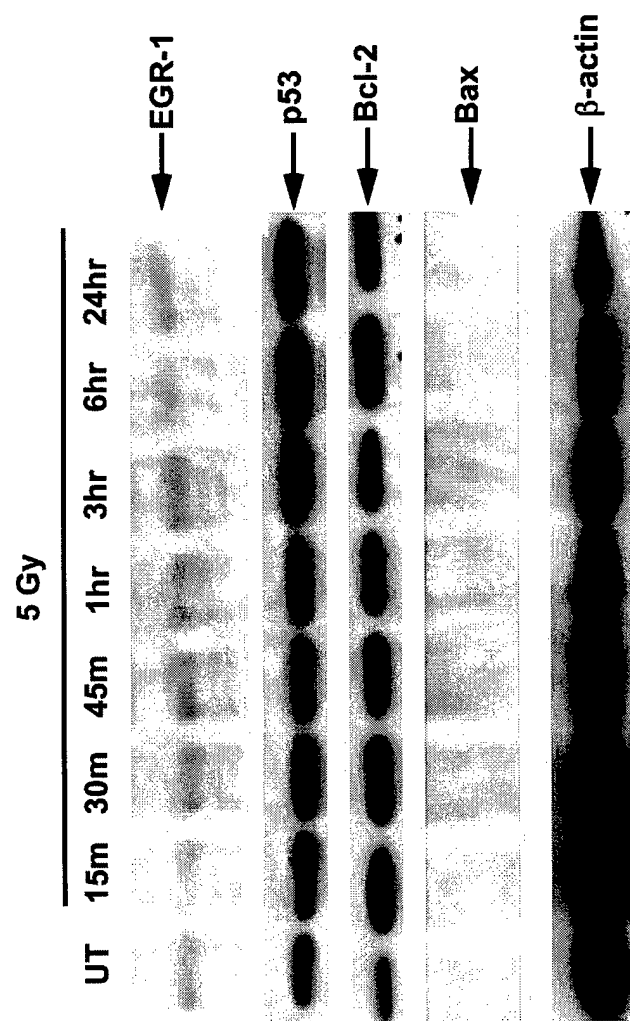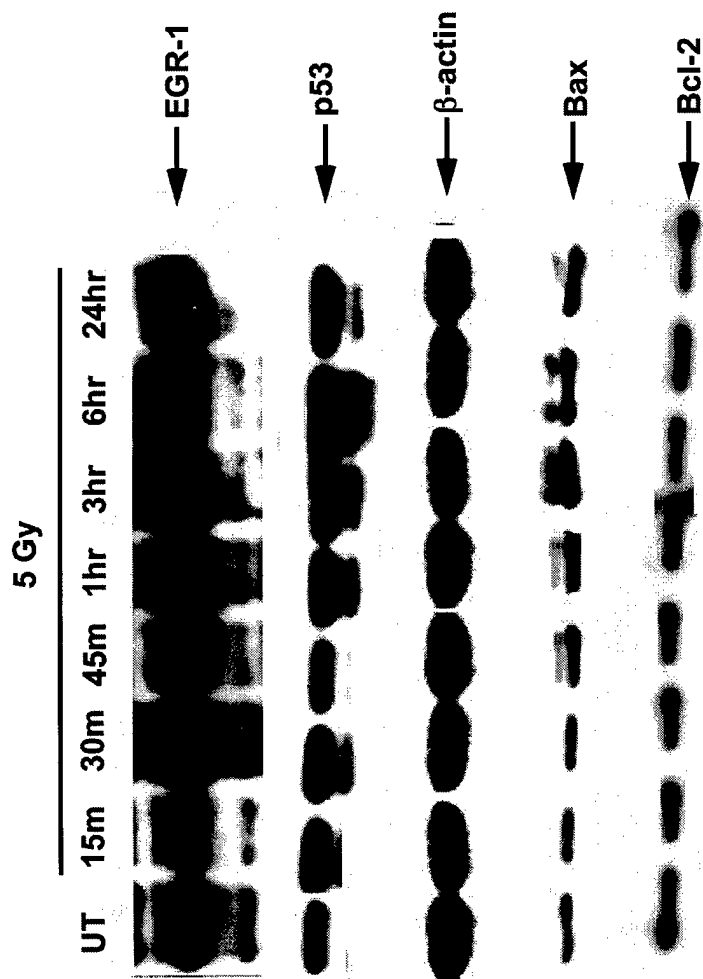
Figure 8. Radiation-induced clonogenic inhibition in PZ-HPV-7 cells infected with
adenoviral vectors containing GFP or EGR-1 or NAB1

PZ-HPV7(Ad: GFP)

PZ-HPV7(Ad: GFP-Nab1)

PZ-HPV7(Ad: GFP-EGR1)

Dose (Gy)

Surviving Fraction

Figure 9. Radiation-induced apoptosis in PZ-HPV-7 infectant cells. The cells were left untreated or irradiated at 5 Gy and TUNEL assay was performed. The untreated levels were normalized with the irradiated groups.

Legend:
- PZ-HPV7(Ad: GFP)
- PZ-HPV7(AD: GFP-NAB1)
- PZ-HPV7(Ad: GFP-EGR1)

y-axis: TUNEL positive cells
x-axis: UT, 24h, 48h

Figure 10A. Western blot analysis of gene expression protein kinetics of PZ-HPV-7 cells infected with Ad/GFP-EGR-1. Whole cell protein extracts were prepared from these infectant cells and were left untreated (UT) or exposed to a 5 Gy dose and incubated for time interval indicated. The blot was probed with an antibody for EGR-1, p53, Bcl-2, Bax or β–actin.
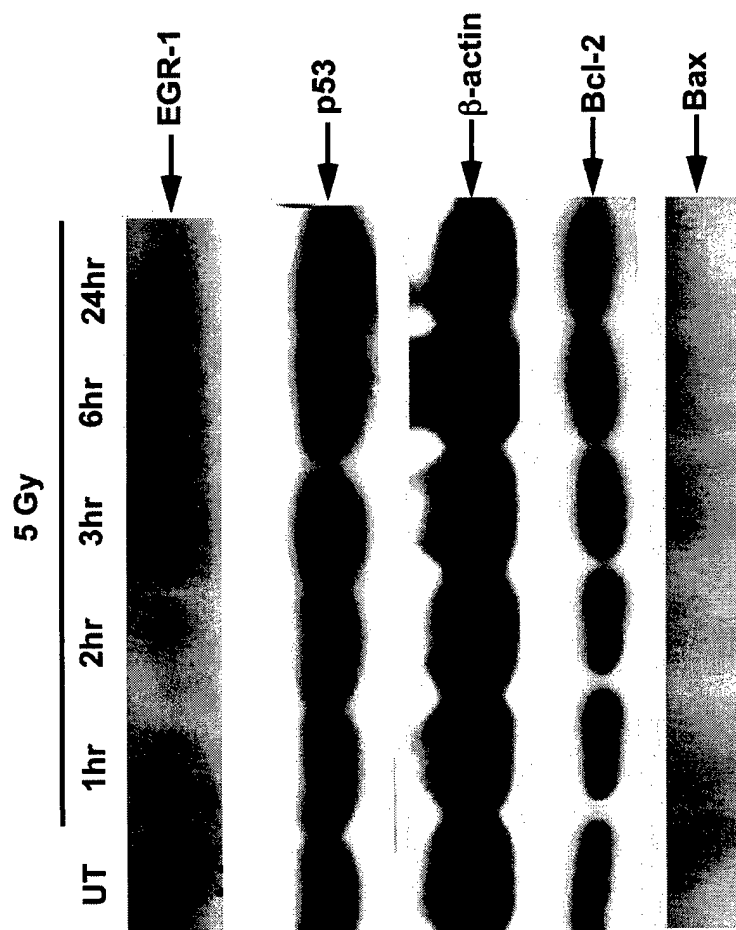
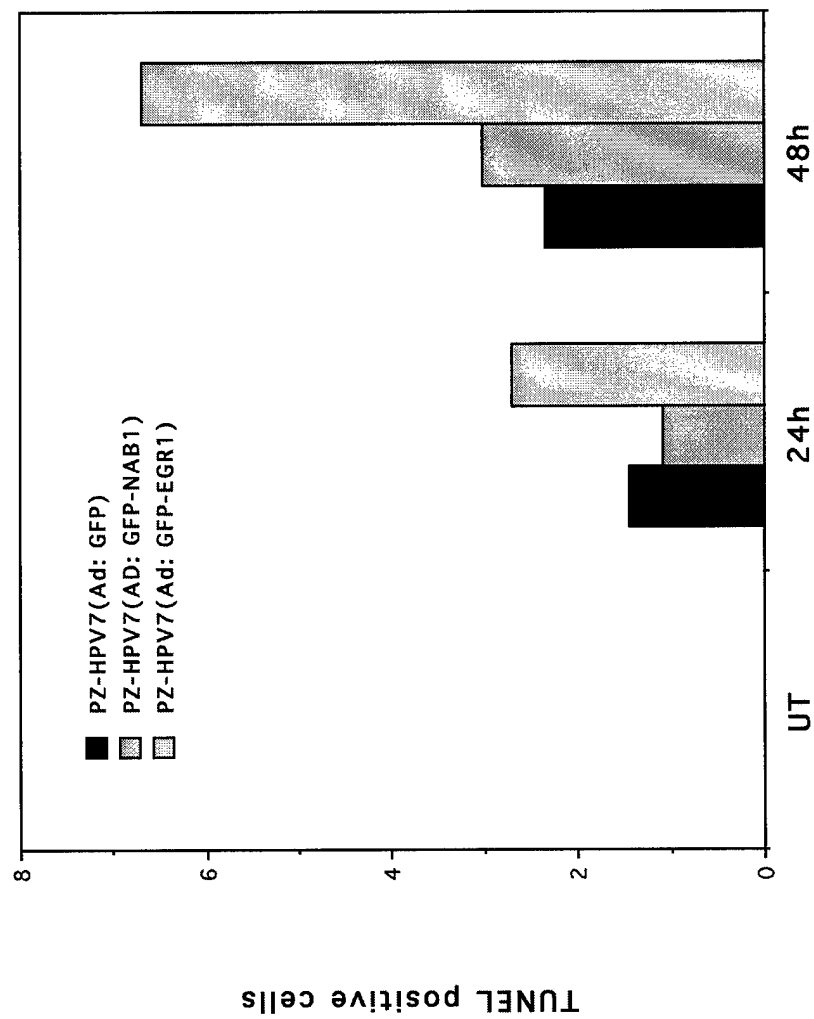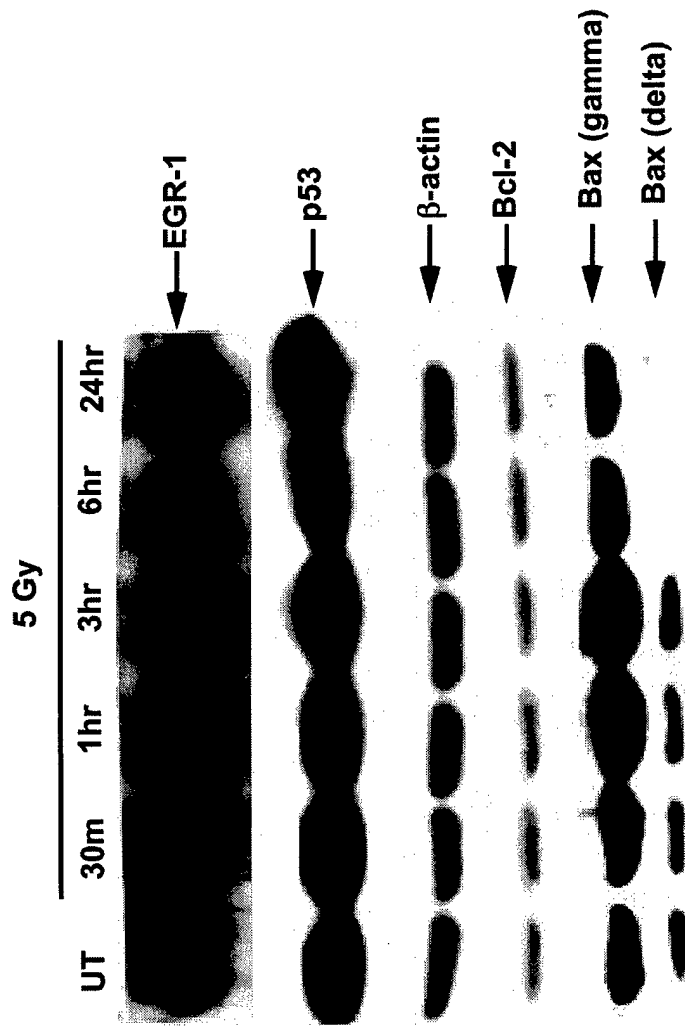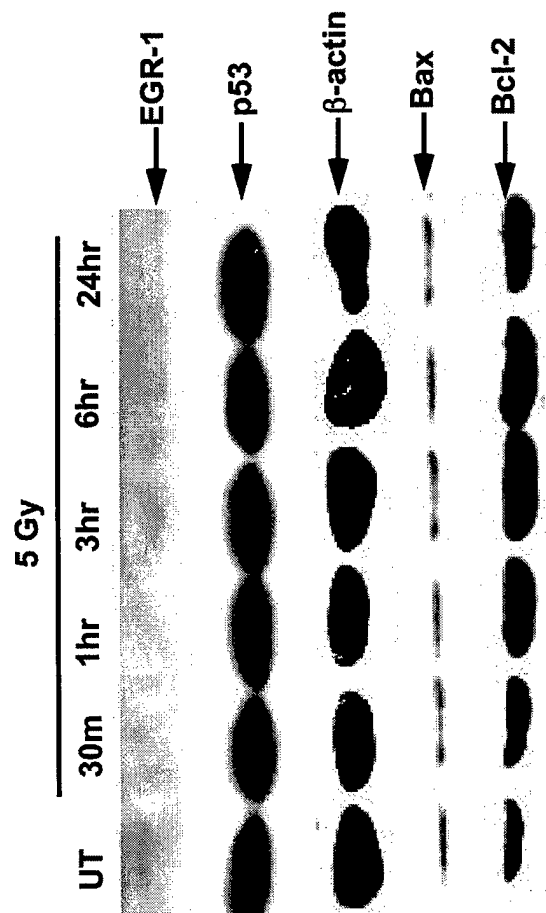Figure 10B. Western blot analysis of gene expression protein kinetics of PZ-HPV-7 cells infected with Ad/GFP-NAB-1. Whole cell protein extracts were prepared from these infectant cells and were left untreated (UT) or exposed to a 5 Gy dose and incubated for time interval indicated. The blot was probed with an antibody for EGR-1, p53, Bcl-2, Bax or β-actin.

Ionizing Radiation Down-regulates p53 Protein in Primary Egr-1$^{-/-}$ Mouse Embryonic Fibroblast Cells Causing Enhanced Resistance to Apoptosis*

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In this study, we sought to investigate the mechanism of the proapoptotic function of Egr-1 in relation to p53 status in normal isogenic cell backgrounds by using primary MEF cells established from homozygous (Egr-1$^{-/-}$) and heterozygous (Egr-1$^{+/-}$) Egr-1 knock-out mice. Ionizing radiation caused significantly enhanced apoptosis in Egr-1$^{+/-}$ cells (22.8%; $p < 0.0001$) when compared with Egr-1$^{-/-}$ cells (3.5%). Radiation elevated p53 protein in Egr-1$^{+/-}$ cells in 3–6 h. However, in Egr-1$^{-/-}$ cells, the p53 protein was down-regulated 1 h after radiation and was completely degraded at the later time points. Radiation elevated the p53-CAT activity in Egr-1$^{+/-}$ cells but not in Egr-1$^{-/-}$ cells. Interestingly, transient overexpression of EGR-1 in p53$^{-/-}$ MEF cells caused marginal induction of radiation-induced apoptosis when compared with p53$^{+/+}$ MEF cells. Together, these results indicate that Egr-1 may transregulate p53, and both EGR-1 and p53 functions are essential to mediate radiation-induced apoptosis. Rb, an Egr-1 target gene, forms a trimeric complex with p53 and MDM2 to prevent MDM2-mediated p53 degradation. Low levels of Rb including hypophosphorylated forms were observed in Egr-1$^{-/-}$ MEF cells before and after radiation when compared with the levels observed in Egr-1$^{+/-}$ cells. Elevated amounts of the p53-MDM2 complex and low amounts of Rb-MDM-2 complex were observed in Egr-1$^{-/-}$ cells after radiation. Because of a reduction in Rb binding to MDM2 and an increase in MDM2 binding with p53, p53 is directly degraded by MDM2, and this leads to inactivation of the p53-mediated apoptotic pathway in Egr-1$^{-/-}$ MEF cells. Thus, the proapoptotic function of Egr-1 may involve the mediation of Rb protein that is essential to overcome the antiapoptotic function of MDM2 on p53.

The apoptotic pathways consist of an early component that includes molecular events specific for an inducer or a group of inducers and of downstream effector components common to diverse apoptotic signals (1). Apoptosis has also been reported in a variety of experimental tumor systems following exposure to radiation (2, 3). Ionizing radiation alters the expression of specific genes, the products of which may contribute to the events leading to apoptotic cell death. Ionizing radiation exposure is associated with activation of certain immediate-early genes that function as transcription factors (4). These include members of jun or fos and early growth response (EGR)[1] gene families (5, 6).

The Egr gene family includes Egr-1 (7), Egr-2 (8), Egr-3 (9), Egr-4 (10), and the tumor suppressor, Wilms' tumor gene product, WT1 (11, 12). The Egr family shows a high degree of homology in the amino acids constituting the zinc finger domain and binds to the same GC-rich consensus DNA sequence (13, 14). The Egr-1 gene product, EGR-1, is a nuclear protein that contains three zinc fingers of the C_2H_2 subtype (15, 16). Structure-function mapping studies on EGR-1 protein suggest that the amino acids constituting the zinc finger motif confer DNA binding function, whereas the NH$_2$-terminal amino acids confer transactivation function (16, 17). More recent studies have found that sequences diverging from the consensus may also bind EGR-1 (18, 19), thus having a broader spectrum of potential target genes. It is interesting to note that within this family of transcription factors, EGR-1 was found to be a positive activator of transcription, whereas WT1 is a transcriptional repressor, both acting via binding to the same GC-rich consensus sequence in reporter constructs (20–22). Depending on the cell type, EGR-1 may behave as a positive or negative regulator of gene transcription (16, 23, 24). The EGR-1 GC-rich consensus target sequence, 5'-GCG(T/G)GGGCG-3' or 5'-TCC(T/A)CCTCCTCC-3' (25), has been identified in the promoter regions of the following: (a) transcription factors, such as MYC and NUR77; (b) growth factors or their receptors, such as transforming growth factor-β1, TNF-α, PDGF-A (26), PDGF-B (27), insulin-like growth factor-II, fibroblast growth factor-β, or epidermal growth factor receptor (6, 7, 28, 29); (c) cell cycle regulators such as the retinoblastoma susceptibility gene Rb (30), cyclin D1 (31), c-Ki-ras (26), and p53 (32); and (d) thymidine kinase, an enzyme crucial in DNA biosynthesis (18) and MDR-1 (33).

It has also been speculated that x-ray induction of PDGF, transforming growth factor-β1, and TNF-α may be regulated by Egr-1 and c-jun (6). Apart from being a potential transcriptional regulator, Egr-1 has a radiation-inducible promoter. Through these distinct induction pathways, Egr-1 has been

[1] The abbreviations used are: EGR, early growth response; Egr-1, early growth response-1 gene; CAT, chloramphenicol acetyltransferase; EBS, EGR-1-binding site; MEF, mouse embryonic fibroblast; Gy, gray; CMV, cytomegalovirus; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; TUNEL, terminal transferase-mediated dUTP-digoxigenin nick end labeling; TNF-α, tumor necrosis factor-α; PDGF, platelet-derived growth factor; Rb, retinoblastoma gene.

TABLE I
Sequences of primers used for ^{32}P RT-PCR analysis

Gene	Position	Primer sequence	GenBank™ accession number
p53	178–188	5'-ACAGTCGGGATATCAGCCTCG-3' (upper)	X01237
	429–453	5'-TTTTTTGAGAAGGGACAAAAGATG-3' (lower)	K01700
mdm-2	260–281	5'-CAGCTTCGGAACAAGAGACTC-3' (upper)	X58876
	550–570	5'-CTGCTCTCACTCAGCGATGT-3' (lower)	
p21	102–123	5'-CCTGGTGATGTCCGACCTGTT-3' (upper)	U24173
	450–470	5'-GGGGAATCTTCAGGCCGCTC-3' (lower)	
bax	7–25	5'-GGGTCCGGGGAGCAGCTT-3' (upper)	L22472
	486–506	5'-GGGGGTCCCGAAGTAGGAG-3' (lower)	
β-Actin	26–45	5'-TGGGCCGCTCTAGGCACCA-3' (upper)	M12481
	247–266	5'-TGGCCTTAGGGTTCAGGGG-3' (lower)	

linked to signaling events initiating cell phenotypic response to radiation injury.

On the other hand, wild type *p53* has been shown to be functionally necessary for growth inhibition and apoptosis following exposure to ionizing radiation, and *p53* mutations have been reported to increase resistance to apoptosis (34). In our previous report, using melanoma cells, which contain wild type p53, a dose-dependent increase in EGR-1 expression with dose-dependent growth inhibition was observed when exposed to ionizing radiation (35). Transfectant melanoma cells stably expressing the dominant-negative mutant protein of EGR-1 showed significantly reduced (<50%) sensitivity to radiation-inducible growth inhibition, and this resistance was found to be dose-dependent. These observations suggest that the EGR-1 induction is involved in the regulation of radiation-inducible apoptosis despite the presence of wild type p53. Recently, we used a p53 null prostate cancer cell line (PC-3), which was found to be moderately resistant to ionizing radiation-inducible apoptosis (36). Western blot analysis and immunocytochemistry studies indicate that EGR-1 is induced in the PC-3 cells by ionizing radiation. Experiments with the *Egr-1* dominant-negative mutant or *Egr-1* overexpression suggest that *Egr-1* function is required for the radiation-inducible apoptosis. Despite the absence of wild type functional p53 protein, the transfected cells expressing the dominant-negative mutant of EGR-1 were resistant to ionizing radiation, and cells overexpressing EGR-1 protein were sensitive to ionizing radiation. Our findings strongly suggested that the radiation-induced apoptotic response in PC-3 cells is elicited through up-regulation of TNF-α protein via EGR-1-mediated transactivation. Thus, EGR-1 is an important mediator of radiation responsiveness irrespective of p53 functional status. However, in a recent report, it was found that EGR-1 protein transactivates the promoter of *p53* gene and up-regulates p53 mRNA and protein levels in response to apoptotic stimuli (32). This prompted us to investigate further the interactive role of *Egr-1* with p53 during the process of apoptosis. We sought to investigate this mechanism in a normal cell background using isogenic normal primary culture cells derived from mouse embryonic fibroblasts (MEF) with varied genomic status for *Egr-1* gene (cells with both intact *Egr-1* alleles, *Egr-1$^{+/+}$*; cells with homozygous deletion of *Egr-1* alleles, *Egr-1$^{-/-}$*; and heterozygous deletion of one *Egr-1* allele, *Egr-1$^{+/-}$*). Based on findings from these isogenic normal cells with varied genomic status of *Egr-1*, we suggest that EGR-1 function is necessary for enhanced sensitivity to radiation-induced apoptosis and that the radiation-induced proapoptotic function of *Egr*-1 is directly mediated by the target genes *p53* and *Rb*.

MATERIALS AND METHODS

Cell Culture—Primary cultures of mouse embryonic fibroblast (MEF) cells from normal mice at passage 3 (kindly provided by Dr. Tyler Jacks, Howard Hughes Medical Institute) labeled as p53$^{+/+}$ were assumed to

have two normal alleles of *Egr-1* wild type (*Egr-1$^{+/+}$*) gene. Primary MEF cultures of cells at passage 0 from homozygous (*Egr-1$^{-/-}$*) and heterozygous (*Egr-1$^{+/-}$*) *Egr-1* knock-out mice (37) were grown in Dulbecco's modified Eagle's medium supplemented with 1% glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin at 37 °C and 5% CO$_2$. Primary MEF cells at passage 3 containing homozygous deletion of wild type *p53* gene (p53$^{-/-}$) established from p53 knock-out mice were also grown in Dulbecco's modified Eagle's medium (kindly provided by Dr. Tyler Jacks, Howard Hughes Medical Institute).

Plasmid Constructs—The plasmid CMV-EGR-1, which encodes full-length EGR-1 protein, contains EGR-1 cDNA downstream of the CMV promoter in the vector pCB6$^+$ (21). Plasmid pCMV-WT1-EGR-1, which encodes a dominant-negative mutant of EGR-1, contains a WT1-EGR-1 chimera downstream of the CMV promoter in the vector pCB6$^+$ (21). The reporter construct, EBS-CAT, contains three EGR-1-binding sites (CGCCCCCGC) placed in tandem upstream of a minimal c-*fos* promoter and CAT cDNA. The p53-CAT construct (pAA-CAT), which contains 337-base pair (*Ava*II-*Ava*II) fragment (−441 to −104) of *p53* promoter placed in front of CAT cDNA (38), was kindly provided by Dr. Moshe Oren, Wiezmann Institute of Science, Israel. The EGR-1-binding site (TCC)$_3$T(TCC) on pAA-CAT was located at −44 to −32. The Rb promoter region from −1343 to −1135 was generated from mouse genomic DNA template by PCR. The sense primer (5'-TTTT<u>TCTAGA</u>CGAGC-CTCGCGGACGTGA-3') and antisense primer (5'-AAAA<u>AAGCTT</u>CAT-GACGCGCACGCGGGC-3') contained built-in-sites (underlined) for *Xba*I and *Hin*dIII, respectively, and they generated a 236-base pair fragment of Rb promoter. The 236-base pair fragment of *Rb* promoter was cloned in pG-CAT, a vector for CAT reporter (Rb-CAT). The control pCAT reporter vector was purchased from Promega.

DNA Transfection and CAT Assays—Transient transfections were performed by the calcium phosphate coprecipitation method as described previously (36). CAT assays were performed by thin layer chromatography as described previously (36).

Irradiation—A 100-kV industrial x-ray machine (Phillips, Netherlands) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm aluminum plus 1-mm beryllium filter was 3.85 Gy at a focus-surface distance of 20 cm.

Quantitation of Apoptosis—Apoptosis was quantified by TUNEL staining and flow cytometry. The ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD), that detects DNA strand breaks by terminal transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) was used as described (36). Briefly, cells were seeded in chamber slides, and the next day they were exposed to a 5-Gy dose of radiation. After 24 h, the DNA was tailed with digoxigenin-dUTP and conjugated with an anti-digoxigenin fluorescein. The specimen was counter stained with propidium iodide and antifade. The stained specimen was observed in triple band-pass filter using Nikon-microphot epifluorescence microscope. To determine the percentage of cells showing apoptosis, four experiments in total were performed, and ~1000 cells were counted in each experiment. For flow cytometry, cells were lifted by using nonenzymatic cell dissociation medium (Sigma), washed with phosphate-buffered saline, stained with Hoechst (Ho342) and merocyanine (MC540), and analyzed by flow cytometry using a FAC-Star Plus cell sorter as described (36).

^{32}P-Reverse Transcriptase-Polymerase Chain Reaction (^{32}P-RT-PCR) of p53 and Its Target Genes—Total RNA was isolated from untreated and irradiated *Egr-1$^{+/-}$* and *Egr-1$^{-/-}$* cells at various time intervals using TRIzol reagent (Life Technologies, Inc.). One μg of total RNA was reverse-transcribed into cDNA using oligo(dT) primers and reverse transcriptase in a 40-μl reaction mix as described previously (35). Radiation-induced mRNA expression of *p53*, p21$^{waf1/cip1}$, *mdm-2*, and

bax were analyzed by PCR. PCR was performed by using the products of reverse transcription reaction and the upstream and downstream primers flanking the *p53*, p21$^{waf1/cip1}$, *mdm-2*, and *bax* genes (Table I) and β-actin gene as an internal control.

Western Blot Analysis—Total protein extracts from untreated and irradiated cells at various time intervals were subjected to Western blot analysis as described (35), using anti-EGR-1 antibody (sc-110) (Santa Cruz Biotechnology), anti-p53 antibody (pAb240) (sc-99), anti-MDM2 antibody (PharMingen 65101A), anti-Rb antibody (PharMingen 14001 A), or for loading control the anti-β-actin antibody (Sigma). The bound immune complexes were detected using the chemiluminescence method (Santa Cruz Biotechnology).

Immunoprecipitation—Cells were lysed with triple detergent lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1% Nonidet P-40, 0.5% sodium deoxycholate), and 1 mg of lysates was incubated with antibodies (either anti-MDM2 antibody, PharMingen 65101A, or goat polyclonal anti-p53 antibody FL-393-G) as indicated. The antibody complexes were isolated using protein A/G-agarose beads (Santa Cruz Biotechnology) and washed three times with phosphate-buffered saline. The immunoprecipitated protein with beads were boiled in SDS sample buffer, and the supernatants were analyzed on SDS-polyacrylamide gel and subjected to Western blot analysis using anti-Rb antibody or anti-MDM2 antibody.

RESULTS

Ionizing Radiation Induces EGR-1 Protein That Transactivates via the GC-rich Binding Site in Egr-1⁺/⁻ and Egr-1⁺/⁺ MEF Cells—To determine whether radiation causes induction of EGR-1 protein in *Egr-1⁺/⁻* and *Egr-1⁺/⁺* MEF cells, whole cell protein extracts were prepared from the cells at different time intervals after exposure to a 5-Gy dose of ionizing radiation and subjected to Western blot analysis. As shown in Fig. 1A, no detectable basal level of EGR-1 protein was found in untreated *Egr-1⁺/⁻* and *Egr-1⁻/⁻* cells. After exposure to 5-Gy dose of radiation, EGR-1 protein was induced at 30 min (10-fold) after the exposure (Fig. 1A) in *Egr-1⁺/⁻* cells. However, this induction was absent in *Egr-1⁻/⁻* cells.

To ascertain the EGR-1-dependent transactivation process in *Egr-1⁻/⁻*, *Egr-1⁺/⁻*, and *Egr-1⁺/⁺* MEF cells, we performed transient transfections with the following: (*a*) only reporter construct EBS-CAT that contains three tandem EGR-1-binding sites; (*b*) EBS-CAT and an EGR-1 expression construct CMV-EGR-1; or (*c*) EBS-CAT and then exposed the cells to ionizing radiation. As seen in Fig. 1B, CAT activity was completely absent in basal and irradiated *Egr-1⁻/⁻* cells, whereas transient transfection with CMV-EGR-1 elevated the CAT activity. In *Egr-1⁺/⁻* and *Egr-1⁺/⁺* cells, ionizing radiation increased the relative CAT activity in an allelic dose-dependent manner. Similarly, *Egr-1⁺/⁺* cells showed slightly higher basal CAT activity as compared with *Egr-1⁺/⁻* cells. However, the CMV-EGR-1 construct caused an increase in CAT reporter activity irrespective of endogenous *Egr*-1 allelic status (Fig. 1B). These results confirmed that the EGR-1 protein is necessary for the transactivation of target genes containing the EGR-1-binding sequence.

Ionizing Radiation Caused Enhanced Cell Death in Egr-1⁺/⁻ Cells—MEFs (*Egr-1⁻/⁻* and *Egr-1⁺/⁻* cells) were left untreated or irradiated at 5-Gy dose of ionizing radiation. TUNEL staining and flow cytometry were performed to determine the incidence of apoptosis. By TUNEL assay, the incidence of apoptosis after 24 h of radiation was 3.5% in *Egr-1⁻/⁻* cells and 22.8% in *Egr-1⁺/⁻* cells (Fig. 2A). By flow cytometry assay using MC540 and Hoechst 342 staining, the incidence of apoptosis after 48 h of radiation was 6.2% in *Egr-1⁻/⁻* cells and 53% in *Egr-1⁺/⁻* cells (Fig. 2B). Thus, ionizing radiation caused significantly enhanced apoptosis in *Egr-1⁺/⁻* cells ($p < 0.0001$) when compared with *Egr-1⁻/⁻* cells as demonstrated by TUNEL and flow cytometry assays. These observations suggest that despite the presence of wild type functional *p53* gene in this normal cell
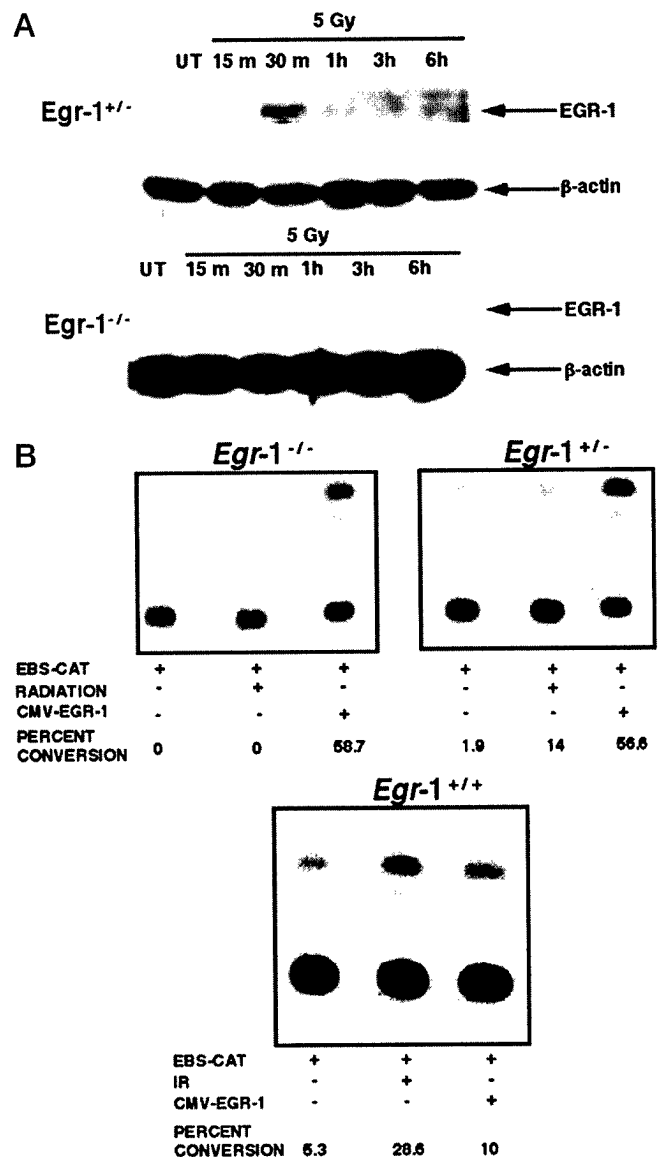

FIG. 1. **EGR-1 is induced by ionizing radiation.** *A*, EGR-1 protein induction detected by Western blot analysis. Whole cell protein extracts were prepared from *Egr-1⁺/⁻* and *Egr-1⁻/⁻* cells that were left untreated (*UT*) or exposed to a 5-Gy dose of ionizing radiation for various time intervals (in hours) and then subjected to Western blot analysis for EGR-1 or β-actin. The blot was subsequently probed with an antibody for EGR-1 or β-actin. *B*, ionizing radiation and CMV-EGR-1 transactivate EBS-CAT reporter construct containing three tandem repeats of EGR-1-binding sites. MEF cells were transiently cotransfected with 4 µg of EBS-CAT or 4 µg of CMV-EGR-1. Next, the cells were either left unexposed or exposed to a 5-Gy dose of ionizing radiation, and CAT activity was assayed and expressed as percent conversion of [¹⁴C]chloramphenicol to acetylated forms.

background, MEFs with homozygous deletion of *Egr*-1 were resistant to ionizing radiation-inducible apoptosis.

High Basal Levels and Lack of Induction of p53, p21$^{waf1/cip1}$, mdm-2, and bax mRNA by Radiation in Egr-1⁻/⁻ Cells—To ascertain whether radiation up-regulates p53 mRNA and the p53 target genes such as p21$^{waf1/cip1}$, *mdm-2*, and *bax*, we performed ³²P-RT-PCR using the RNA extracted from untreated and irradiated cultures at various time points. In *Egr-1⁺/⁻* cells, p53 mRNA was elevated after 15–30 min of irradiation (Fig. 3). Both p21$^{waf1/cip1}$ and *mdm-2* were elevated in 30 min (Fig. 3); *bax* was also elevated up to 1 h of exposure to ionizing radiation in *Egr-1⁺/⁻* cells (Fig. 3). However, in *Egr-1⁻/⁻* cells, no up-regulation was evident for *p53*, p21$^{waf1/cip1}$, *mdm-2, and bax* genes (Fig. 3). The basal levels of *p53*,

FIG. 2. **Radiation-induced apoptosis in** *Egr*-1$^{+/-}$ **and** *Egr*-1$^{-/-}$ **MEF cells.** *A,* quantification of apoptosis by TUNEL assay. Apoptosis was quantified by TUNEL. To determine the percentage of cells showing apoptosis, a total of 1000 cells were counted for each experiment. Background levels in untreated cells were normalized over those in treated cells. Data represents a mean of two experiments. The *error bars* represent S.D. *B,* quantitation of apoptosis by Hoechst 33342 (*Ho342*) and merocyanine 540 (*MC540*) staining. Cells were irradiated at 5 Gy and after 48 h stained with Hoechst 33342 and merocyanine 540. The gates were set so as to analyze cell cycle and apoptosis stages as described previously (37). Hoechst 33342 is a DNA-specific dye that measures DNA content, and merocyanine 540 bind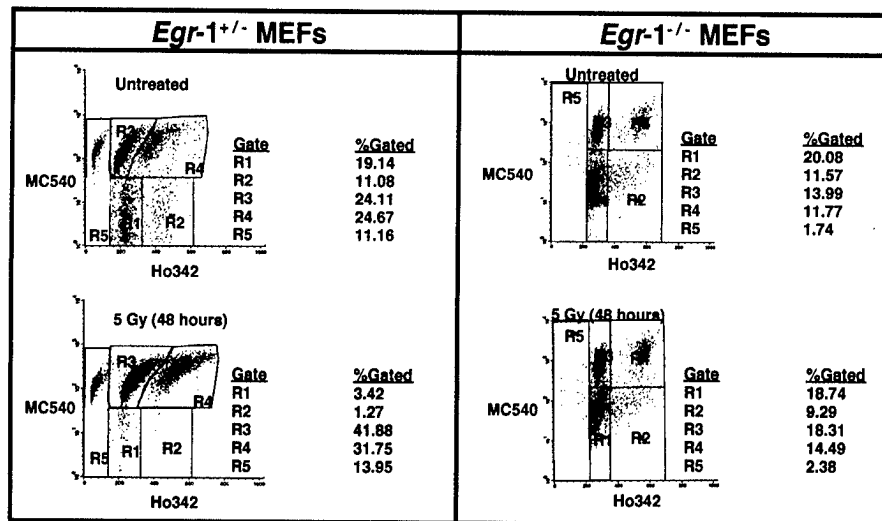s to membrane phospholipids that are exposed on the outside of the membrane during the process of apoptosis. These two dyes separate five distinct populations of tumor cells as follows: viable resting cells, 2n DNA content and merocyanine 540-unstained (R1); viable cycling cells, >2n DNA content and merocyanine 540-unstained (R2); viable resting cells undergoing apoptosis, 2n DNA content and merocyanine 540-stained (R3); viable cycling cells undergoing apoptosis, >2n DNA content and merocyanine 540-stained (R4); and late stage apoptotic cells that are merocyanine 540-stained but Hoechst 33342-unstained indicating DNA fragmentation (R5). The data shown are representative of two independent experiments. The untreated population contained merocyanine 540-stained cells owing to spontaneous apoptosis that occurred during cell culture. The percent increase (mean ± S.D. from two experiments) in apoptotic cells (*i.e.* merocyanine 540-stained cells in the R3, R4, and R5 compartments) in the irradiated population over the untreated population was 6.18 ± 1.02 in *Egr*-1$^{-/-}$ cells and 52.97 ± 2.32 in *Egr*-1$^{+/-}$ cells.

A

B

p21$^{waf1/cip1}$, *mdm-2,* and *bax* were higher in *Egr*-1$^{-/-}$ cells as compared with *Egr*-1$^{+/-}$ cells. Recently, it was reported that EGR-1 protein directly binds to Rel homology domain in p65 (Rel A) subunit of NFκB complex (39), and p65 was found to transactivate the *p53* promoter (40, 41). High basal levels of p53 and its target genes in *Egr*-1$^{-/-}$ MEFs (Fig. 3) may be attributed to induction of *p53* promoter by elevated NFκB activity in the absence of *Egr*-1 function. Thus, absence of induction of these genes after radiation may have contributed to enhanced radiation resistance in *Egr*-1$^{-/-}$ cells.

Ionizing Radiation Caused Down-regulation of p53 Protein in Egr-1$^{-/-}$ MEF Cells—Western blot analysis was performed to examine whether exposure to ionizing radiation caused induction of p53 protein. *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ cells were either left untreated or exposed to a 5-Gy dose of ionizing radiation, and proteins were extracted at various time intervals and subjected to Western blot analysis for p53 protein. As seen in Fig. 4, a strong induction of p53 protein was noticed in *Egr*-1$^{+/-}$ cells after irradiation; after 3–6 h of radiation, p53 protein levels were increased about 5-fold in *Egr*-1$^{+/-}$ cells. However, in *Egr*-1$^{-/-}$ cells, the p53 protein was down-regulated after 1 h of radiation and reduced to <10% of basal levels at 6- and 12-h time points (Fig. 4). The above observations have ascertained the fact that EGR-1 protein is necessary to cause radiation-induced apoptosis. Absence of EGR-1 protein renders enhanced resistance to radiation. Thus, the loss of p53 protein in *Egr*-

$1^{-/-}$ cells after radiation may have contributed to enhanced resistance to apoptosis.

Transfection of CMV-EGR-1 in Egr-1$^{-/-}$ Cells Led to Restoration of Sensitivity to Radiation-induced Apoptosis—To understand the regulation of p53 by *Egr*-1, we transiently transfected *Egr*-1$^{-/-}$ cells by using CMV-EGR-1 or vector-alone constructs. Transiently transfected cells were left untreated and irradiated, and then either total proteins were extracted for Western blot analysis of p53 protein or TUNEL was performed at 24 or 48 h after irradiation. In *Egr*-1$^{-/-}$ cells transfected with vector alone, p53 levels were down-regulated after radiation (Fig. 5A), and these cells showed 8 or 11% cell death at 24 or 48 h after radiation, respectively (Fig. 5B). On the other hand, *Egr*-1$^{-/-}$ cells transfected with CMV-EGR-1 showed no down-regulation of p53 protein (Fig. 5A) and 10 or 38% cell death at 24 or 48 h after radiation, respectively (Fig. 5B).

We further performed p53-CAT reporter assays to understand whether the down-regulation of p53 protein after radiation was due to loss of EGR-1-mediated transactivation in *Egr*-1$^{-/-}$ cells. Basal and irradiated p53-CAT reporter activity was examined in *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ MEF cells. Both MEF cells showed basal p53-CAT activity; however, higher CAT activity was observed in *Egr*-1$^{+/-}$ cells than *Egr*-1$^{-/-}$ cells (Fig. 5C). Radiation elevated the p53-CAT activity in *Egr*-1$^{+/-}$ cells, whereas radiation caused no change in the p53-CAT activity in
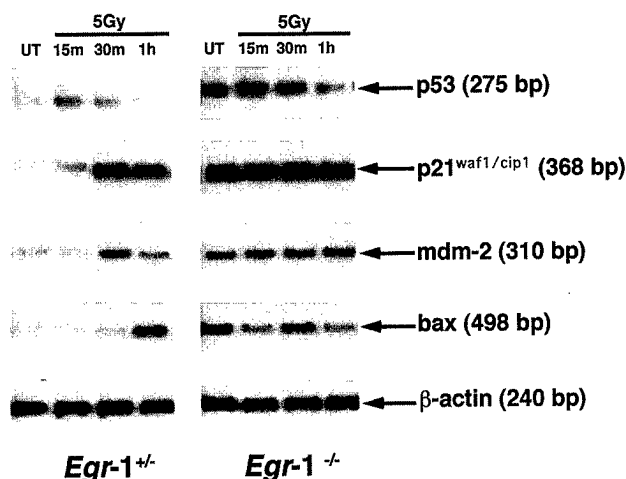
FIG. 3. **RT-PCR analysis of *p53*, p21$^{waf1/cip1}$, *mdm-2*, and *bax* in untreated or irradiated *Egr*-1$^{+/-}$ or *Egr*-1$^{-/-}$ MEF cells.** *Egr*-1$^{+/-}$ or *Egr*-1$^{-/-}$ MEF cells were left untreated (*UT*) or treated with radiation (5 Gy), and total RNA was extracted at the indicated time points. PCR was performed by using the products of reverse transcription reaction and the upstream and downstream primers flanking the *p53*, p21$^{waf1/cip1}$, *mdm-2*, and *bax* genes (Table I) and β-actin gene as an internal control. *bp*, base pair.
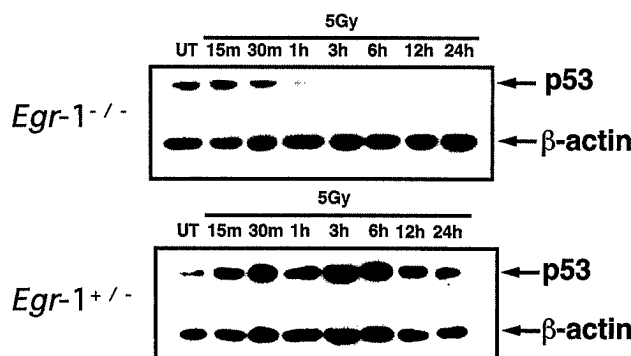
FIG. 4. **p53 protein is down-regulated by ionizing radiation in *Egr*-1$^{-/-}$ cells.** Protein levels of p53 were detected by Western blot analysis before and after radiation. Whole cell protein extracts were prepared from *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ cells that were left untreated (*UT*) or exposed to a 5-Gy dose and incubated for the time interval indicated and then subjected to Western blot analysis for p53 or β-actin.

Egr-1$^{-/-}$ cells (Fig. 5*C*). These results indicate that *Egr*-1 may transregulate p53. Together, *Egr*-1 is pivotal to mediate the apoptotic action by ionizing radiation.

Overexpression of CMV-EGR-1 in p53$^{-/-}$ MEF Cells Led to Modest Induction of Radiation-induced Apoptosis—Our data in Fig. 5*C* indicated lack of p53 promoter activity after radiation in *Egr*-1$^{-/-}$ cells, and this prompted us to understand precisely the cooperative role of *Egr*-1 and p53 in the regulation of radiation-induced apoptosis. We transiently transfected p53$^{+/+}$ and p53$^{-/-}$ MEF cells by using CMV-EGR-1 or CMV-WT1-EGR-1 (dominant-negative mutant of EGR-1) or vector pCB6$^+$ constructs. Transiently transfected cells were left untreated and irradiated, and then TUNEL was performed 24 and 48 h after radiation (Fig. 6).

In p53$^{+/+}$ MEF cells, overexpression of EGR-1 caused significant induction of cell death after 48 h when compared with p53$^{+/+}$ cells overexpressing vector alone. However, p53$^{+/+}$ cells overexpressing dominant-negative mutant EGR-1 showed reduction in cell death when compared with p53$^{+/+}$ cells overexpressing vector alone (Fig. 6*A*).

Interestingly, overexpression of EGR-1 protein in p53$^{-/-}$ MEF cells caused modest induction of cell death after 48 h of

FIG. 5. **Restoration of radiation sensitivity and stabilization of p53 protein in *Egr*-1$^{-/-}$ MEF cells transiently transfected with CMV-EGR-1.** *A*, protein levels of p53 were detected by Western blot analysis before and after radiation. Whole cell protein extracts were prepared from vector-transfected *Egr*-1$^{-/-}$ cells or CMV-EGR-1-transfected *Egr*-1$^{-/-}$ cells that were left untreated (*UT*) or exposed to a 5-Gy dose and incubated for the time interval indicated and then subjected to Western blot analysis for p53 or β-actin. *B*, radiation-induced apoptosis in *Egr*-1$^{-/-}$ transfectants was determined by TUNEL assay. Transfectants were left untreated (*UT*) or irradiated at 5-Gy dose of radiation and subjected to TUNEL analysis. Approximately 1000 cells in total were scored for TUNEL-positive cells in each experiment. The data shown here are the percent TUNEL-positive cells as a function of irradiation. *C*, absence of *Egr*-1-mediated transactivation in transiently transfected p53-CAT reporter construct in irradiated *Egr*-1$^{-/-}$ MEF cells. *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ cells were transiently transfected with 4 μg of p53-CAT reporter plasmid. Transfected cells were left untreated or irradiated at 5 Gy, and CAT activity was assayed and normalized by determining the percent conversion of [^{14}C]chloramphenicol to acetylated forms using densitometric ratios.

FIG. 6. **Radiation-induced apoptosis depends on both functionally active EGR-1 and p53 proteins.** Radiation-induced apoptosis was quantified by TUNEL assay using vector or CMV-EGR-1 or CMV-WT1-EGR-1-transfected p53$^{+/+}$ (A) and p53$^{-/-}$ (B) MEF cells. Transfectant cells were left untreated (*UT*) or irradiated at 5-Gy dose of radiation and after 24 and 48 h were subjected to TUNEL analysis. Approximately 1000 cells in total were scored for TUNEL-positive cells in each experiment. Data shown are percent TUNEL-positive cells as a function of irradiation dose. Data represents a mean of three experiments. The *error bars* represent S.D.

FIG. 7. **Lack of hypophosphorylated form of Rb protein leads to MDM2-mediated p53 degradation in Egr-1$^{-/-}$ cells.** A, protein levels of Rb and MDM2 were detected by Western blot analysis before and after rad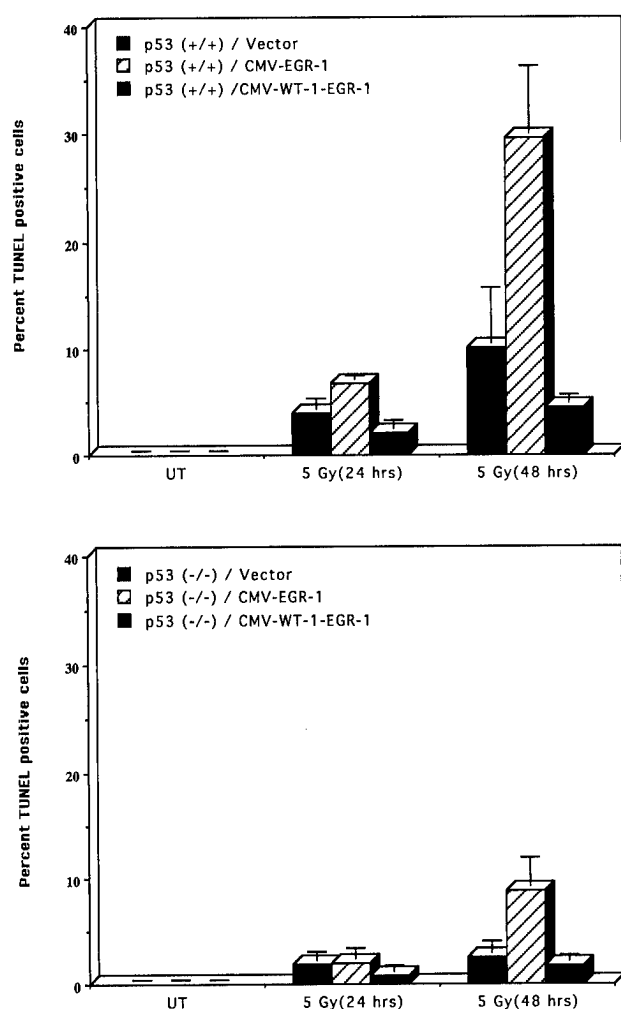iation. Whole cell protein extracts were prepared from Egr-1$^{+/-}$ and Egr-1$^{-/-}$ cells that were left untreated (*UT*) or exposed to a 5-Gy dose and then after 1 h of incubation were subjected to Western blot analysis for Rb, MDM2, or β-actin. Expression of β-actin was used as loading control. *B,* absence of *Egr*-1-mediated transactivation in transiently transfected Rb-CAT reporter construct in irradiated *Egr*-1$^{-/-}$ MEF cells. *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ cells were transiently transfected with 4 μg of p53-CAT reporter plasmid. Transfected cells were left untreated or irradiated at 5 Gy, and CAT activity was assayed and normalized by determining the percent conversion of [^{14}C]chloramphenicol to acetylated forms using densitometric ratios. *C,* Rb forms a complex with p53 through MDM2. Cell lysates were prepared from *Egr*-1$^{+/-}$ and Egr-1$^{-/-}$ cells that were left untreated (*UT*) or exposed to a 5-Gy dose, and after 1 h of incubation were subjected to immunoprecipitation. The bound complexes of Rb, p53, and MDM2 was detected by coprecipitating Rb with MDM2 antibody and coprecipitating MDM2 with p53 antibody and subjected to Western blot analysis.

radiation when compared with p53$^{-/-}$ MEF cells overexpressing vector alone (Fig. 6B). Overexpression of dominant-negative mutant EGR-1 in p53$^{-/-}$ MEF cells showed reduction in radiation-induced cell death when compared with cells overexpressing vector alone. These data strongly suggest that both functional EGR-1 and p53 are essential to mediate radiation-induced apoptosis; however, absence of p53 may not contribute toward complete abrogation of EGR-1-mediated radiation-induced apoptosis.

Lack of a Hypophosphorylated Form of Rb Protein Led to MDM2-mediated p53 Degradation in Egr-1$^{-/-}$ Cells—Recently, it was reported (30) that Rb regulates the stability and the apoptotic function of p53 via MDM2 (42). It is also known that EGR-1 regulates Rb through its consensus site on the *Rb* promoter. Since Rb critically regulates the stability of p53 protein, we hypothesized that the degradation of p53 protein after radiation in *Egr*-1$^{-/-}$ cells may due to loss of *Egr*-1-mediated transactivation of *Rb* gene. To test this hypothesis, we performed the following experiments to understand the mechanism of p53 degradation in irradiated *Egr*-1$^{-/-}$ cells.

First, to ascertain EGR-1-mediated induction of Rb, we per-

formed Western blot analysis for Rb protein expression levels and Rb-CAT reporter assays in untreated and irradiated *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ MEF cells. Western blot analysis showed low levels of Rb including hypophosphorylated forms in *Egr*-1$^{-/-}$ MEF cells before and after radiation when compared with *Egr*-1$^{+/-}$ cells (Fig. 7A). Rb-CAT reporter assay also indicated low basal CAT activity in *Egr*-1$^{-/-}$ cells when compared with *Egr*-1$^{+/-}$ cells (Fig. 7B). After radiation, Rb-CAT activity was elevated to 2-fold in *Egr*-1$^{+/-}$ cells but not in *Egr*-1$^{-/-}$ cells (Fig. 7B). Recent studies have demonstrated that MDM2-p53 interaction directly targets p53 degradation (43). Since p53 was

degraded in irradiated *Egr*-1$^{-/-}$ cells, we performed Western blot analysis to determine the levels of MDM2 in untreated and irradiated *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ MEF cells. Interestingly, MDM2 levels were down-regulated after radiation in *Egr-1$^{-/-}$* cells; on the other hand, MDM2 levels were up-regulated after radiation in *Egr-1$^{+/-}$* cells (Fig. 7A).

Low levels of hypophosphorylated forms of Rb and low levels of MDM2 after radiation were evident in *Egr*-1$^{-/-}$ cells when compared with *Egr*-1$^{+/-}$ cells. Based on these observations, we hypothesized that p53 degradation in irradiated *Egr*-1$^{-/-}$ cells might be due to the presence of higher amounts of p53-MDM2-bound forms and relatively lower amounts of Rb bound to the p53-MDM2 complex (trimeric complex of Rb-MDM2-p53). To test this hypothesis, we performed immunoprecipitation experiments followed by Western blot analysis with cell lysates from untreated and irradiated *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ MEF cells. Radiation caused high levels of Rb-MDM2 complex relative to p53-MDM2 complex in *Egr*-1$^{+/-}$ cells (Fig. 7C). By contrast, higher amounts of p53-MDM2 complex and lower amounts of Rb-MDM-2 complex were observed in *Egr-1$^{-/-}$* cells after radiation (Fig. 7C). Thus, the degradation of p53 in *Egr-1$^{-/-}$* cells after radiation may be due to diminished Rb binding to MDM2 and enhanced MDM2 binding to p53. Because of diminished Rb binding to MDM2, p53 is directly degraded by MDM2, and thus the p53-mediated apoptotic pathway in *Egr-1$^{-/-}$* MEF cells is inactivated.

DISCUSSION

Exposure to ionizing radiation is associated with the formation of reactive oxygen intermediates causing direct damage to DNA (44). These reactive oxygen intermediates target the sequence $CC(A/T)_6GG$ to mediate the activation of EGR-1 (4). Previous studies from our laboratory (35) have suggested that despite the presence of wild type p53 background, inhibition of the expression or function of EGR-1 causes a diminution of radiation-induced growth inhibition in melanoma cells. In the absence of p53, radiation-induced apoptosis of prostate cancer cells was found to be mediated by EGR-1 via TNF-α transactivation (36). These results suggest that *Egr*-1 induction is involved in the radiation-induced signaling of the cascades of apoptosis pathway.

In this study, in contrast to *Egr*-1$^{+/-}$ MEF cells, *Egr*-1$^{-/-}$ MEF cells were significantly resistant to radiation-inducible apoptosis and showed no elevation of p53 protein after radiation. These observations indicate that radiation-induced EGR-1-mediated transactivation of downstream genes is essential for radiation sensitivity. Thus, in support of previous reports, the present study demonstrates that EGR-1 is the upstream mediator for the initiation of the radiation-induced signaling cascade leading to cell death.

The tumor suppressor gene *p53* is a central mediator of apoptotic pathways in diverse model systems (45–48). The p53 protein can cause transcriptional up-regulation of a number of downstream genes, such as *mdm-2*, p21$^{waf1/cip1}$, *bax, fas/apo1*, insulin-like growth factor-binding protein-3, which are implicated in growth inhibition and apoptotic cell death (46–48). In this study, it was found that mRNA levels of *p53*, p21$^{waf1/cip1}$, *mdm-2,* and *bax* were elevated after irradiation in *Egr*-1$^{+/-}$ cells but not in *Egr*-1$^{-/-}$ cells. In addition, the basal levels of these mRNAs were high in *Egr*-1$^{-/-}$ MEF cells when compared with *Egr*-1$^{+/-}$ cells. Loss of radiation-induced elevation of p53 may be attributed to the loss of *Egr*-1 mediated transregulation of *p53* in *Egr*-1$^{-/-}$ MEF cells, and this may have led to the loss of up-regulation of *p53* target genes, p21$^{waf1/cip1}$, *mdm-2*, and *bax*.

Radiation caused degradation of p53 protein in *Egr*-1$^{-/-}$ cells, and this led to enhanced resistance to radiation-inducible apoptosis. Transient overexpression of EGR-1 protein in *Egr*-

1$^{-/-}$ cells restored radiation sensitivity and stabilized the p53 protein levels. Thus, this observation suggests that EGR-1 protein is necessary for the up-regulation and the stability of p53 protein and radiation sensitivity. Moreover, radiation elevated the p53-CAT reporter activity in *Egr*-1$^{+/-}$ cells but not in *Egr*-1$^{-/-}$ cells. This observation is supported by a recent study that EGR-1 can directly bind with the *p53* promoter at two consensus EGR-1-binding sites and induce the p53 mRNA and protein (32). Thus, *Egr*-1 is an important transregulator of p53. However, at this point we cannot rule out the possibility that other genes that are also regulated by *Egr*-1 may play a role in the stability of p53 protein.

A marginal induction of radiation-induced apoptosis observed in p53$^{-/-}$/CMV-EGR-1 MEF transfectants when compared with p53$^{+/+}$/CMV-EGR-1 MEF cells suggests that p53 played an important downstream role in regulation of *Egr*-1-mediated radiation-induced apoptosis. It also suggests that the absence of p53 may not contribute toward complete abrogation of EGR-1-mediated radiation-induced apoptosis. This is supported by our previous data that in p53 null prostate cancer cell line PC3, EGR-1 overexpression caused super induction of radiosensitivity (36). The degree of induction of apoptosis was much higher in p53 null PC3 cells when compared with p53$^{-/-}$/CMV-EGR-1 MEF transfectant cells in this study. The difference may be due to the tumor cell background *versus* the normal cell background. Thus, in the absence of p53, EGR-1 may mediate the proapoptotic action of radiation via TNF-α (36) or other downstream cell-death effector genes.

p53 can bind to the promoter region of MDM2 and activate its transcription, forming an autoregulation loop between the expression and function of p53 and MDM2 (49). It is also reported that MDM2-p53 interaction can target p53 for degradation (43). Rb can regulate the apoptotic function of p53 through binding to MDM2, thus preventing MDM2-mediated degradation of p53 (42). Rb can also prevent MDM2 from inhibiting p53-mediated apoptosis. In addition, Rb can protect p53 from MDM2-mediated degradation by forming a trimeric complex with p53 via binding to MDM2 (42). To understand further the mechanism of p53 degradation in irradiated *Egr*-1$^{-/-}$ MEF cells, we investigated the expression and functional interaction of Rb with p53 and MDM2 in *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ MEF cells. The rationale for analyzing the Rb function in this normal isogenic cell system is that (*a*) Rb regulates the apoptotic function of p53 by mitigating MDM2 mediated degradation (42) and (*b*) the Rb gene promoter contains EGR-1-binding sites that conform to the GC-rich consensus (30). Low expression levels of hypophosphorylated forms of Rb and decreased Rb-CAT reporter activity were found in *Egr*-1$^{-/-}$ MEF cells before and after irradiation when compared with *Egr*-1$^{+/-}$ MEF cells. Relatively higher levels of Rb-MDM2-bound complex and lower levels of p53-MDM2-bound complex were observed in irradiated *Egr*-1$^{+/-}$ MEF cells. In contrast, higher amounts of p53-MDM2 complex and low bound forms of the Rb-MDM2 complex were observed in *Egr*-1$^{-/-}$ cells. Lower amounts of the Rb-MDM2 complex along with higher amounts of p53-MDM2 in *Egr*-1$^{-/-}$ MEF cells might have contributed to p53 degradation after radiation. Thus, apoptosis caused by ionizing radiation requires the induction of EGR-1 protein, which then transregulates the expression of p53 protein and also indirectly regulates the stability of p53 via Rb.

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Early Growth Response-1 gene: a potential radiation response gene marker in prostate cancer[¶]

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ABSTRACT

This study was undertaken to determine whether the transcription factor EGR-1 expression (1) in the primary tumor correlates with radiation response in terms of complete local tumor control with no evidence of disease or recurrence / evidence of metastasis; (2) in the post-irradiated biopsies correlates with residual tumor; and (3) correlates with the expression of *Egr*-1 target genes such as p53, pRB and Bax. We analyzed (a) 25 pretreated surgically resected paraffin-embedded primary adenocarcinomas of the prostate for the presence of the EGR-1 expression and mutation and correlated with clinical end-points such as serum PSA levels and current clinical status; (b) 27 post-irradiated biopsies of prostate for the presence of EGR-1 expression and to correlate these findings to the residual tumor status; and (c) 12 prospective prostate tumor specimens for EGR-1 expression and its target genes. EGR-1 expression was determined by immunohistochemistry and mutations were screened in two regions of *Egr*-1 gene (trinucleotide AGC repeats in transactivation domain (TD) and poly A tract in 3'UTR) by PCR-SSCP analysis. Out of 25 patients, 18 patients showed expression of EGR-1. Nuclear staining was weak or usually absent in non-malignant regions. Poorly differentiated carcinoma showed more intense nuclear staining than moderately differentiated tumors. Out of 18 patients with EGR-1 expression, five had mutation in the transactivation domain and one had mutation in both transactivation and 3'UTR region. EGR-1 overexpression correlated with treatment failure. Additionally, in post-irradiated biopsy specimens, 8 of 9 cases with residual tumor (18 out of 27 were benign) showed intense EGR-1 staining only in the carcinoma. No correlation with EGR-1 overexpression and its' target genes were found which may indirectly suggest that overexpressed EGR-1 may lack transactivation function. In summary, EGR-1 overexpression in the mutant form may provide an indication of clinical failure (local recurrence or metastasis).

KEYWORDS

Prostate cancer, radiation, EGR-1, gene overexpression, radiation resistance and apoptosis.

INTRODUCTION

Tumor aggressiveness correlates with enhanced resistance to apoptosis. Treatment strategies such as chemotherapy and radiation eliminate malignant cells by the induction of apoptosis as well as by "mitotic death". For example, therapeutic ionizing radiation can cause DNA strand breakage or distortion of the DNA nucleoprotein conformation which may transduce signals that result in activation of early response genes (*c-jun*, *c-fos* and *Egr*-1) whose gene products may then stimulate later genes such as TNF-α, IL-1 and PDGF-α. These later genes are important in cellular response to radiation injury (such as cell death). Therefore, it is to the advantage of tumor cells to acquire mutation and overexpression of these cellular genes that protect against these processes.

Prostate cancer has been treated by radiation therapy for many years with a wide range of radiation types and methods of delivery. Radiation therapy is administered in at least four clinical situations: (i) for the treatment of primary cancer; (ii) immediately after radical prostatectomy either because of pathologic findings in the final surgical specimen or because of an elevated PSA level; (iii) at the time of a clinically diagnosed, palpable local recurrence; or (iv) after the diagnosis of metastatic cancer in the pelvic lymph nodes [1].

Changes inducible by radiation therapy in prostate cancer are highly variable among patients and also in different areas of the tumor within the same patient [2]. The radiation-inducible change is characterized by a decrease in the number of cancer glands, with these being haphazardly arranged in the tissue. There is also a evident change in histologic grade. Bostwick et al [3] reported a change in the Gleason score of 2 or greater in 7 of 26 post radiation biopsies and similar findings of significant increase in Gleason score were documented by another group [4]. Clinically recurring local tumors after radiation therapy are frequently of a much higher

4

histologic grade and this most likely represents progression through clonal evolution [5]. These histologic changes following radiation therapy possibly reflect a dedifferentiation phenomena [3], with poor radiation response, the histologic grade and the tissue architecture remains unchanged.

The clinical significance of histologically positive tumor after radiation therapy is of paramount interest. The rate of positive biopsies after radiation therapy has varied from 20% [6] to 93% [7], with the majority of the series reporting in the 50 to 60% range [8-10]. The significance of positive biopsies appeared to correlate with an increased risk of distant metastases [9-11] and death from prostate cancer [12,13]. In early studies, it was documented that the frequency of positive biopsies decreased with an increase in time after completion of radiation therapy [3,8,14], although this was not uniformly observed [15,16]. It is also interesting to note that some patients with positive biopsies showed no evidence of local progression [12]. Crook *et al* [17] applied proliferating cell nuclear antigen (PCNA) immunostaining to positive post-radiation therapy biopsy specimens from 83 patients and observed a significantly higher progression rate in PCNA positive biopsies (53%) than those that were PCNA negative (22%).

The molecular basis of these histologic changes and clinical outcome is not known. Because 50 to 60% of prostate cancer patients have histologically positive biopsies [6,7] after radiation therapy with an increased risk of local recurrence, distant metastases and death, the status of molecular events in these positive histological biopsies needs to be elucidated. Thus, analysis of molecular indicators of cell proliferation or programmed cell death (apoptosis) will be of value in identifying patient subsets, which may require possible radiation dose escalation in order to achieve complete local control of prostate adenocarcinoma. We selected the transcription factor *Egr*-1 as a molecular indicator of radiation response because EGR-1 is necessary for radiation-induced apoptosis [18-21] and it is overexpressed in majority of human

prostate tumors [22,23]. Here, we report that overexpression of EGR-1 protein in prostate tumors

potentially in mutant form (lacking transactivating ability) correlates with treatment failure.

METHODS

Patients and tumor specimens

In our study, we analyzed 25 pretreated surgically resected paraffin-embedded primary

adenocarcinomas of the prostate for the presence of the EGR-1 expression and mutation; 27 post-

irradiated biopsies of prostate for the presence of EGR-1 expression; and 12 prospective

surgically resected or biopsied prostate tumor specimens for expression EGR-1 and its' target

genes.   These randomly selected patients were admitted to the Chandler Medical Center,

University of Kentucky between 1993 and 2001 for the treatment of prostate cancer. The study

was approved by the Institutional Review Board of the University of Kentucky. Clinical data

was obtained following completion of the immunohistochemical study. The prostatic

adenocarcinomas were graded according to the Gleason grading scheme [24]. Pathologic staining

was according to the guidelines of the American Joint Committee on Cancer [25,26].

Immunohistochemical (IHC) Staining

Sections from each case were stained with the following antibodies: anti-EGR-1 antibody

(sc-110), anti-p53 antibody (D0-1), anti-Bax antibody (sc-7480) (Santa Cruz Biotechnology,

CA) and anti-pRB antibody (14001A) (PharMingen). The avidin-biotin-peroxidase complex

method of staining was used for immunohistochemical staining [27]. The IHC assay was scored in

a semiquantitative approach utilizing both the intensity and distribution of specific staining. The

staining intensity (I) was graded as 0 (if no staining was observed), 1 (weak staining), 2

(moderate staining) or 3 (strong staining). The proportion (P) of cells (0 - 1.0) with the observed

staining intensity was recorded. A score (so called histologic or H-score) for each case was determined as the product of intensity and proportion (H=I x P) [27].

Isolation of DNA and ^{32}P-PCR-SSCP analysis of *Egr*-1 gene

Ten sections of paraffin-embedded tissue blocks of 4.5 micron size (for each sample, new blade was used to avoid any carry-over contamination) was thoroughly mixed in xylene in a 1.5 ml eppendorf tube for 20 minutes. Xylene was decanted after centrifugation at 1200 rpm. Xylene wash was repeated twice and final wash was performed with 100% ethanol. The tissue was dried and digested in extraction buffer containing 50mM Tris pH 7.5, 10mM EDTA, 1% sodium dodecyl sulphate and 500 µg/ml of Proteinase K (Sigma, St. Louis, USA) at 37°C overnight. DNA was precipitated with 2.5 M sodium acetate and absolute ethanol after phenol-chloroform de-proteination extraction. The precipitated DNA was dissolved in 10 µl of DNase/RNase free water and the whole amount was used for 50 µl PCR amplification.

PCR was performed using ^{32}P-end labeled 3' and 5' primers flanking "AGC" trinucleotide repeat region in the transacitivation domain (between nucleotides 454 to 522) (5'-ATGGACAACTACCCTAAGCTGGAGG-3', sense primer and 5'-GGTGCTCGTAGGGCTGCTC- 3', anti-sense primer) and "A" mononucleotide repeat region in the 3'untranslated region (between nucleotides 2401 to 2427) (5'-TTGGGGTACTCTTGATGTGAA-3', sense primer and 5'-GCATCATCACAAAATAGAGGA-3', anti-sense primer) of the *Egr*-1 gene [28]. Mutations in these regions were screened by Single Strand Conformation Polymorphism (SSCP) analysis as described previously [18,29].

RESULTS

Overexpression of EGR-1 in prostate tumors correlates with treatment failure

Results of EGR-1 protein expression, mutation and other clinico-pathological findings for pretreated specimens are shown in Table 1. Out of 25 patients, 18 patients showed expression of EGR-1 (Figure 1). Predominantly, EGR-1 protein was found in the nucleus of basal cell of prostatic acini. Nuclear staining was weak or usually absent in non-malignant regions. Poorly differentiated carcinoma showed the most intense nuclear staining than moderately differentiated tumors (Figure 1). There was no correlation with the Gleason scores. Out of 18 patients with EGR-1 expression, five had mutation in the transactivation domain and one had mutation in both transactivation and 3'UTR region (Figure 2). As compared to EGR-1 overexpression, the incidence of mutation may be underrepresented since a very small area of the gene was analyzed. Information on the clinical outcome of the disease in 7 out 18 EGR-1 positive patients were not available. Out of 11 positive cases with EGR-1 expression, five patients failed (out of which two had mutation) with either clinical recurrence of disease (two cases) or biochemical failure (three cases). No statistically significant correlation was found between the expression of EGR-1 protein and the initial stage, Gleason grade or PSA level. Results of treatment indicated that 9 patients treated with radical prostatectomy whose tumors expressed EGR-1 protein, four (45%) developed local recurrence as compared to one out of four with negative expression for EGR-1 (not statistically significant). Of the 7 patients treated with radiation, two out of three (66%) with EGR-1 expression recurred as compared with none out of four patients negative for EGR-1 expression. These results indicate that prostate tumors overexpress EGR-1 protein and overexpression of the EGR-1 protein potentially in the mutant form may provide an indication of treatment failure.

Residual tumor in post-irradiated biopsies show intense staining of EGR-1 protein

Paraffin-embedded prostate biopsy specimens were obtained from 27 patients after 4 to 6 weeks of hyperfractionated radiation therapy. Out of 27 biopsies analyzed, 18 were all benign, 2 were all cancer and 7 had benign and cancer regions. Out of 18 benign cases, 6 showed sporadic EGR-1 staining, mostly in the basal cells. Out of 2 cases with cancer one showed EGR-1 staining (a H-score of 2). And all the 7 cases with benign and cancer region, showed EGR-1 staining in the cancer areas only (a H-score of 1 to 3) (Figure 3). Thus, 8 out 9 residual tumors showed intense EGR-1 staining only in the cancer regions, suggesting that aberrant expression of EGR-1 protein may implicate failure to radiation therapy.

EGR-1 overexpression in prostate tumors does not cause an increase *Egr*-1 target gene expression.

Egr-1 is strong transcription factor and regulates transcription of genes involved in growth inhibition and cell death pathways [20]. We previously reported that the radiation induced pro-apoptotic function of EGR-1 is directly mediated by the target genes p53, Rb [20] and TNF-α genes. Our recent analysis also demonstrated that overexpression of wild EGR-1 protein in prostate cancer cell line DU-145 directly transactivates Bax causing an imbalance in Bcl-2:Bax protein ratio and rendering enhanced susceptibility to radiation-induced apoptosis [21]. Thus, by assessing the expression of Egr-1 target genes and correlating with the EGR-1 expression status may indirectly reveal the transactivation ability of overexpressed EGR-1 in prostate tumors. To test this, we analyzed 12 prostate tumor specimens for EGR-1 expression and it's target genes p53, pRB and Bax. Eleven out of 11 cases showed EGR-1 overexpression (H-score of 1 to 3) (Table 2) and one case was not evaluated due to lack of tissue material in the slide. For p53 expression, 10 out of 12 cases were evaluated in which 7 out of 10 showed expression of p53

protein (H-score of 0.3 to 1.7). For pRB expression, 8 out of 10 cases showed expression of pRB protein (H-score of 0.03 to 1). For Bax expression, 11 out of 12 cases were evaluated in which 6 out 10 showed expression of Bax protein (a H-score of 0.5 to 1.8). No significant correlation between EGR-1 overexpression and elevation of p53 or pRB and Bax was found. Instead, EGR-1 overexpression at a H-score of 3 showed absence of p53 or bax expression (case #4), suggesting that overexpressed EGR-1 protein in these tumors may potentially be mutant and lack transactivation ability.

DISCUSSION

This study indicated that the incidence of EGR-1 overexpression is high in the prostate adenocarcinoma. This is supported by a previous report that high levels of *Egr*-1 mRNA expression was detected in 12 out of 12 intraprostatic adenocarcinomas by PCR and differential display assays [22]. Recently, a study reported by Eid et al indicated that EGR-1 mRNA was expressed at significantly higher levels in cancer than in normal prostate [23]. Our recent study in androgen independent prostate cancer cell line DU-145, showed three alleles of *Egr*-1 gene with elevated basal levels when compared to other prostate cancer cell lines PC-3 and LNCaP [21]. No evidence of mutation was detected in the coding region of *Egr*-1 gene in DU-145 cells and interestingly the endogenous EGR-1 protein showed low transactivation ability in response to radiation [21]. DU-145 cells were highly resistant to radiation-induced apoptosis. In prostate tumor samples, we found a similar situation in which most tumors showed elevated levels of EGR-1 expression and this expression correlated with treatment failure or recurrence as demonstrated in post-irradiated biopsies. Thus, EGR-1 overexpression potentially in non-functional form may be a marker of resistance to therapy.

It is important to note that, in *Egr*-1 gene, the transactivation domain harbors six trinucleotide AGC repeats at two regions in nucleotide positions 454-471 and 505-522 ($AGC_{6/33/6}$) and the 3'UTR has polyadenines at nucleotides 2407 to 2422 (3'UTR A_{16}). The transactivation domain activates transcription 100 fold and the 3'UTR region may have a role in mediating selective mRNA degradation of *Egr*-1 [30]. Mutations in the transactivation domain may abrogate the transcriptional activity. It has been mentioned that, for example, changes in the 3'UTR in *fos* gene do not affect the function of *fos* protein but only the stability of *fos* mRNA is altered [31]. This structural pattern implies that the *Egr*-1 gene may particularly be subject to microsatellite instability (MIN) because of nucleotide repeat sequences in three exon regions. High incidence of MIN (43% to 65%) has been reported in prostate tumors [32,33]. Based on this characteristic behavior of this tumor, these repeat sequences in the *Egr*-1 gene may make it a favorable target for MIN-directed mutation in prostate cancer and may cause accumulation of mutated EGR-1 protein. Interestingly, our analyses in these two regions showed low incidence of mutation, suggesting that overexpression of EGR-1 protein with no obvious mutation in these repeat regions may be due to gene amplification or loss of transactivation potential as observed in prostate cancer cell line DU-145. Our analysis of p53, pRB and Bax gene expression (*Egr*-1 target genes) showed no correlation of elevation with overexpression of EGR-1 protein implying that the overexpressed EGR-1 protein in prostate tumors may lack transcriptional activity.

Thus, the presence of EGR-1 overexpression in prostate tumors may be potentially due to many reasons. A mutation in the transactivation domain may lead to the loss of transcriptional activity of *Egr*-1 and eventually shut off the downstream gene induction pathway (for eg via p53 or TNF-α) that normally leads to growth arrest or cell death caused by various treatment protocols. Or, if *Egr*-1 gene is amplified and if the tumor contains *p53* mutation, then

11

overexpressed wild-type EGR-1 protein may drive mutant *p53* gene causing the cell to be highly resistant to DNA-damaging agents. Or, if *Egr*-1 gene is amplified and lacks its transcriptional activity (as seen in DU-145) and this will eventually shut the *Egr*-1 mediated pro-apoptotic signaling leading to enhanced resistance to apoptosis. In conclusion, based on the small number of samples studied, EGR-1 overexpression (potentially in mutant form or in amplified form or loss of *Egr*-1 transcriptional activity) may provide an indication of clinical failure.

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FIGURE LEGENDS

Figure 1. EGR-1 gene expression in prostate cancer. (A) Radical prostatectomy sample of patient # 5. (a) Upper left showing H&E staining of the tumor at magnification x100 and upper right showing same area of this tumor magnified at 40x. (b) Lower left showing EGR-1 staining in the tumor cells (x100) and the same tumor magnified at x400 (lower right) showing strong nuclear immunoreaction for EGR-1 in the neoplastic cells.

Figure 2. Autoradiograph of PCR-SSCP analysis of the *Egr*-1 5' (transactivation domain-$AGC_{6/33/6}$) and 3'(3'UTR-A_{16}) regions using retrospective tumor samples of prostate cancer. Mutations in transactivation domain were detected. Tumors from PS-49 and PS-54 showed mutation in transactivation domain.

Figure 3. EGR-1 expression in post-irradiated biopsy. The carcinoma cells show intense EGR-1 nuclear staining (40x).

TABLES

Table 1. EGR-1 protein expression, mutation and other clinico-pathological findings in

pretreated tumor specimens obtained from prostate cancer patients.

CODE	GRADE	Treatment		Egr-1			STATUS
				Mutation		Expression	
		Surgery	RT	5'	3'	H-score	
PS-05	7	RP	For Rec	M	N	3	Recurrence
PS-06	7	RP	For Rec	F	F	0	NED
PS-15	4	T	D	N	N	3	Metastasis
PS-19	5	T	D	M	M	3	NED
PS-22	6	T	D	N	N	0	NED
PS-25	7	RP	A	N	N	0	NED
PS-32	8	RP	A	N	N	0	NED
PS-40	7	RP	-	N	N	1	NA
PS-41	7	RP	-	M	N	3	NED
PS-42	6	RP	-	M	N	3	Recurrence
PS-43	7	RP	-	N	N	3	NED
PS-44	7	RP	-	N	N	3	Recurrence
PS-45	7	RP	-	N	N	3	NED
PS-46	6	RP	-	N	N	0	NED
PS-47	8	RP	-	N	N	3	Recurrence
PS-48	6	RP	-	N	N	3	NED
PS-49	6	RP	-	M	N	2	NA
PS-50	6	RP	-	N	N	2	NA
PS-51	6	RP	-	N	N	2	NA
PS-52	8	RP	-	N	N	0	NA
PS-53	6	RP	-	N	N	0	NA
PS-54	8	RP	-	M	N	3	NA
PS-55	8	RP	-	N	N	1	NA
PS-56	PIN	RP	-	N	N	0	NA
PS-57	PIN	RP	-	N	N	0	NA

Grade: Gleason score; PSA: serum PSA levels at the presentation of disease; NED: no

evidence of disease; Surg: Surgery; RT: radiation therapy; RP: radical prostectomy; For

rec: radiation therapy for recurrence; T: TURP; PIN: prostatic intra-epithelial neoplasia;

D: definitive; A:adjuvant; H-score 1 to 3 (see materials and methods); N: Normal for

mutation; M:mutated for *Egr*-1; F: failed to amplify product in PCR assay.

Table 2. Expression of EGR-1 and its target genes p53, pRB and Bax in prospective tumor specimens of prostate cancer.

Case#	Gleason Grade	Pathologic Stage	Treatment	EGR-1	p53	pRb	Bax
1	3 + 4 = 7	NA	Radiation	1	0	0.6	0.9
2	2 + 2 = 4	NA	Radiation	3	0.9	0.3	0.8
3	3 + 3 = 6	NA	Radiation	3	1	1	-
4	4 + 3 = 7	T2cN0	Hormone + Surgery+ Radiation	3	0	0.03	0
5	3 + 3 = 6	T4b N0	Hormone + Surgery+ Radiation	2	0.4	0	1.8
6	3 + 3 = 6	NA	Surgical (prostatectomy)	1	0.6	0	0
7	5 + 4 = 9	NA		-	-	0.2	1.7
8	4 + 3 = 7	T2b N0	Surgical (prostatectomy)	1.5	1.5	0.2	1
9	3 + 4 = 7	T3 N0	Surgical (prostatectomy)	1.1	0.3	0.2	0
10	3 + 4 = 7	T2a N0	Surgical (prostatectomy)	1.5	0	0.2	0
11	4 + 3 = 7	T3a N0	Surgical (prostatectomy)	1	-	0.1	0.7
12	4 + 4 = 8	T2 N0	Surgical (prostatectomy)	1	1.7	0.2	0.5

-, no score (tumor tissue markedly reduced in size following technical processing)
NA: not known (pathologic material limited to biopsies)

Figure 1

Ahmed et al

TRANSACTIVATION DOMAIN

1 2 3 4 5 6 7 8 9

1	:	PS-49
2	:	PS-50
3	:	PS-51
4	:	PS-52
5	:	PS-53
6	:	PS-54
7	:	PS-55
8	:	PS-56
9	:	PS-57

3'UTR (poly A$_{16}$)

P 1 2 3 4 5

1	:	PS-15
2	:	PS-19
3	:	PS-22
4	:	PS-25
5	:	PS-32

Figure 2

Ahmed et al
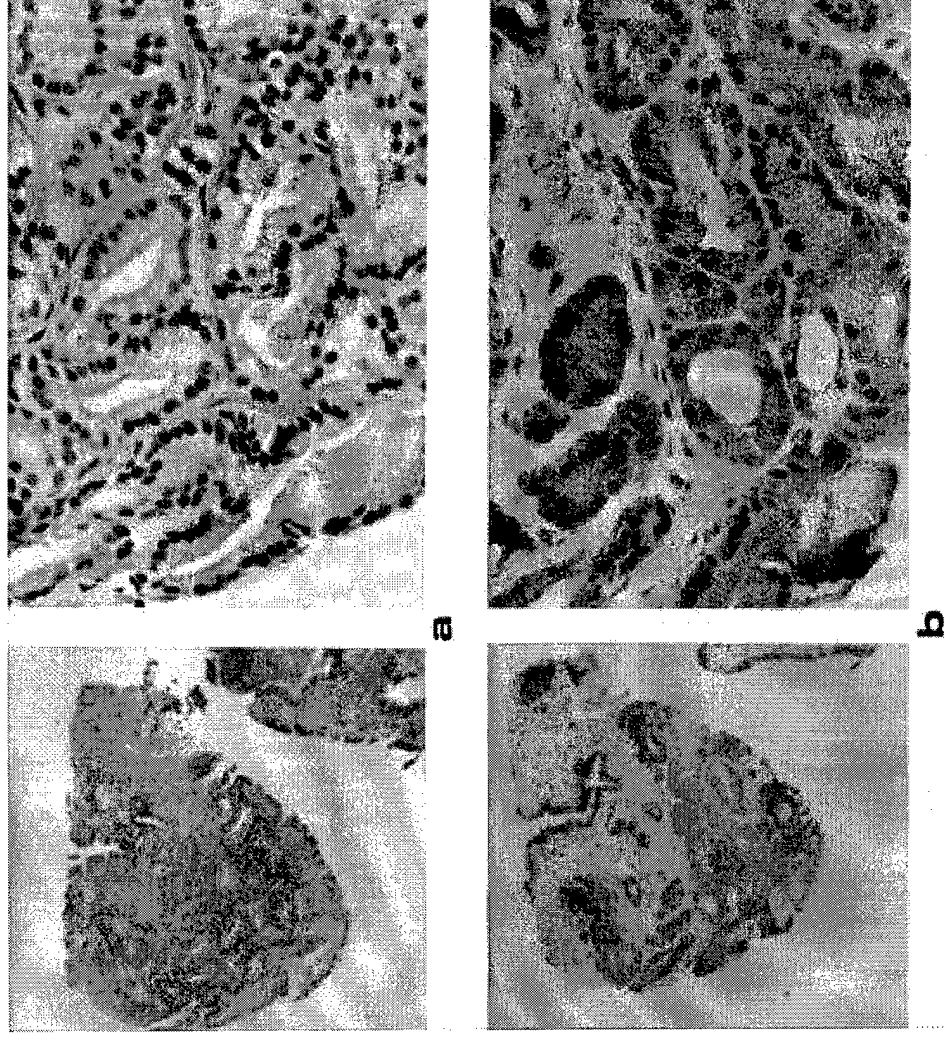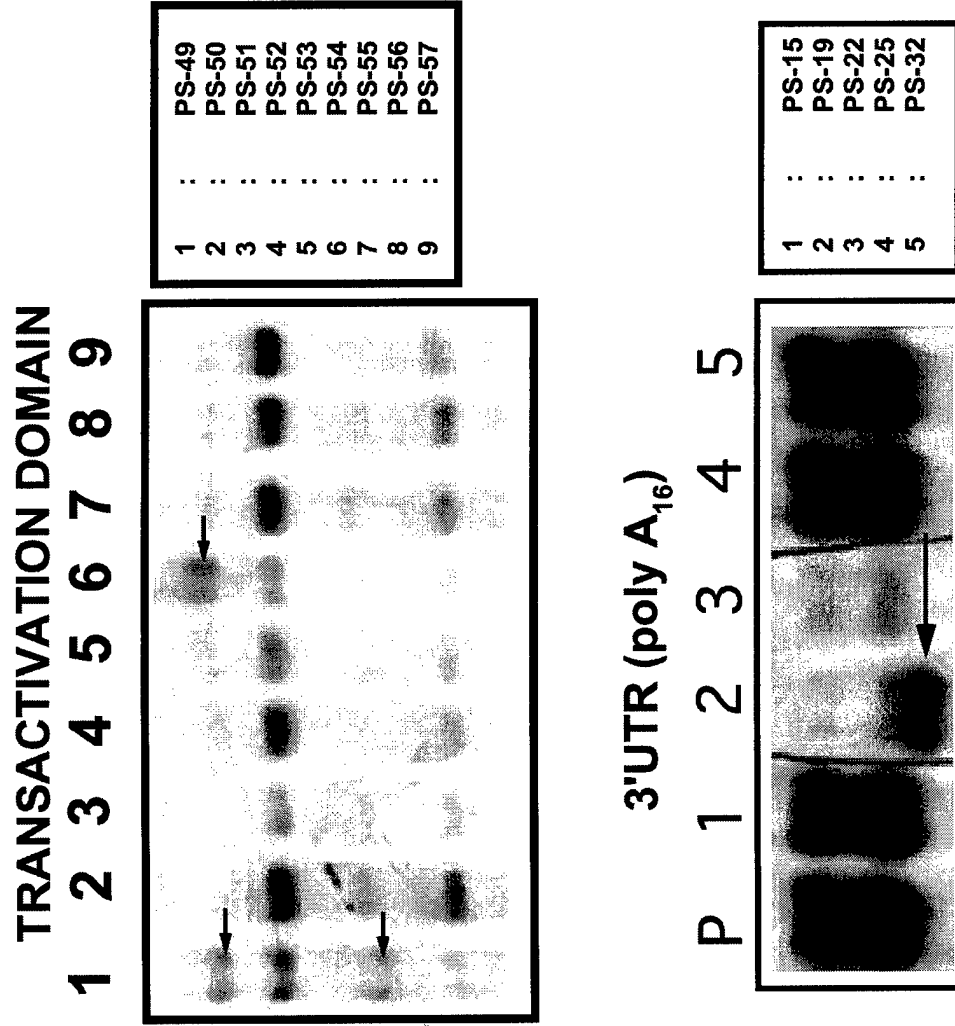

Figure 3

EGR-1 overexpression triggers caspase activation and cell death through transcriptional upregulation of Bax in irradiated p53 mutant prostate cancer cell line DU 145[†]

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Running Title: EGR-1 activates Bax in prostate cancer cells

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ABSTRACT

Early growth response-1 gene (*Egr*-1) is a strong transcriptional activator of key genes involved in growth-inhibition and cell death pathway. In this study, we investigated the functional role of EGR-1 in the regulation of radiation-induced clonogenic inhibition and apoptosis in p53 mutant prostate cancer cell line DU145. The clonogenic assays indicated that the DU145/CMV-EGR-1 cells (SF_2 =0.34; D_0=164 cGy) were significantly sensitive to radiation (p<0.0001) and the dominant-negative mutant of EGR-1 transfected cells DU145/CMV-WT1-EGR1, (SF_2=0.66; D_0=509 cGy) were resistant to radiation (p<0.001) when compared to DU145/Vector cells (SF_2=0.609; D_0=400 cGy). TUNEL analysis also showed significant induction of radiation-induced apoptosis in DU145/CMV-EGR-1 transfectant cells, than DU145/Vector cells alone. Diminished induction of radiation-induced apoptosis was evident in DU145/CMV-WT1-EGR1 cells when compared to DU145/Vector cells alone. Enhanced radiation sensitivity of DU145/CMV-EGR-1 cells was associated with up-regulation of Bax at the RNA and protein level. Radiation also caused down regulation of Bcl-2 in DU145/CMV-EGR-1 cells when compared to unaltered levels of Bcl-2 in DU145/Vector and DU145/CMV-WT1-EGR1 cells. In addition, significant activation of Caspase-3 and Caspase-9 with increased PARP cleavage was observed in DU145/CMV-EGR-1 cells when compared to DU145/Vector or DU145/CMV-WT1-EGR1 cells. Gel shift analysis and CAT reporter assay indicated that EGR-1 transactivates the promoter of the Bax gene that contains two overlapping GC-rich EGR-1 binding sites. These findings establish that radiation-induced proapoptotic action of EGR-1, in a mutant p53 background, directly transactivates Bax that alters the Bcl-2: Bax ratio resulting in significant activation of caspases and induction of cell death pathway.

INTRODUCTION

Carcinoma of the prostate, the most frequently diagnosed cancer in American males, continues to show a steady increase in the annual incidence of newly diagnosed cases [Greenlee, 2000 #64]. It is estimated that 180,400 new prostate cancer cases were diagnosed and the estimated deaths were recorded at 31,900 [Greenlee, 2000 #64]. This tumor is characterized by a remarkably variable, often prolonged natural history [Iczkowski, 1999 #65]. Five-year survival rates for patients with prostate cancer range from 88% for those with localized disease to 29% for those with metastatic disease. Radiotherapy for the treatment of prostate cancer is a well established method of treatment. Recent data indicate that 50-60% of patients have persistent local disease inspite of high dose radiation. A major reason for failure to eradicate local tumor despite high dose radiation is the intrinsic radiation resistance of some tumors.

The clinical significance of histologically positive tumor after radiation therapy is of paramount interest. The rate of positive biopsies after radiation therapy has varied from 20% [Forman, 1993 #66] to 93% [Kabalin, 1989 #67], with the majority of the series reporting in the 50 to 60% range [Mollenkamp, 1975 #68]; [Kurth, 1977 #69]; [Kiesling, 1980 #70]. The significance of positive biopsies appear to correlate with an increased risk of distant metastases [Kurth, 1977 #69]; [Kiesling, 1980 #70]; [Miller, 1993 #71[Scardino, 1986 #72]; [Freiha, 1984 #73] and death from prostate cancer [Scardino, 1986 #72]; [Freiha, 1984 #73].

The molecular basis of these histologic changes and clinical outcome is not known. Analysis of molecular indicators of cell proliferation or programmed cell death (apoptosis) will be of value in identifying patient subsets, which may require possible radiation dose escalation in order to achieve complete local control of prostate adenocarcinoma. In this study, we selected *Egr*-1 gene as a molecular indicator to understand the radiation inducible signal transduction pathway leading either to growth arrest or cell death in prostate cancer cells. We specifically chose *Egr*-1 because of the

following reasons: (a) In normal prostate, upon orchiectomy (castration), *Egr*-1 was rapidly induced and this lead to apoptosis of androgen-dependent cells [Buttyan, 1988 #36]; [Day, 1993 #33]; (b) In most cell type, ionizing radiation upregulates EGR-1 expression and ; (c) EGR-1 protein is a strong transcriptional activator of key genes involved in cell death pathway [Ahmed, 1997 #40]; [Nair, 1997 #35]; [Das, 2000 #61]. Gene expression studies in castrates suggest that transcription factors, such as *Egr*-1 (also referred as NGF-IA) and c-*fos*, may have functional roles in the regulation of the patterns of gene expression upon hormone withdrawal in prostate cell death [Buttyan, 1988 #36]; [Day, 1993 #33]. Another study indicated an association of *Egr*-1 and c-*fos* mRNA expression with apoptosis following 12-tetradecanoyl phorbol 13 acetate (TPA) exposure to androgen-sensitive prostate cells, suggesting a role for Egr-1 mediated transcriptional regulation of apoptosis [Day, 1994 #37]

Wild type p53 has been shown to be functionally important for radiation-induced clonogenic inhibition and apoptosis. Alterations in p53 function has been attributed to increased resistance to radiation induced clonogenic inhibition and apoptosis [Lee, 1993 #38]. Recently, it was reported that EGR-1 protein transactivates p53 gene and up-regulates p53 mRNA and protein levels in response to thapsigargin-induced apoptotic stimuli [Nair, 1997 #35]. To further understand the regulatory role of EGR-1 on p53 function in radiation-induced apoptosis, we performed studies using (a) p53 wild type melanoma cells, (b) p53-null prostate cancer cells and (c) isogenic MEF cells with varied genomic status for *Egr*-1 (*Egr*-1$^{+/-}$ and *Egr*-1 $^{-/-}$ cells). Melanoma cells, containing wild type p53, showed a dose-dependent increase in EGR-1 expression corresponding to radiation-induced growth inhibition [Ahmed, 1996 #39]. While transfectant melanoma cells stably expressing the dominant-negative mutant protein of EGR-1 showed a dose-dependent resistance to radiation-induced growth inhibition. PC3 cells, p53 null prostate cancer cell line, was found to be moderately resistant to ionizing radiation inducible apoptosis [Ahmed, 1997 #40]. In these cells,

EGR-1 was induced after exposure to ionizing radiation. Despite the absence of p53 protein, the transfected cells expressing the dominant-negative mutant of EGR-1 were resistant to ionizing radiation and cells overexpresing EGR-1 protein were sensitve to ionizing radiation. Our findings strongly suggested that the radiation-induced apoptotic response in PC-3 cells was found to be elicited through upregulation of TNF-a protein via EGR-1 mediated transactivation [Ahmed, 1997 #40]. As opposed to our previous reported studies that was performed in prostate cancer cell lines [Ahmed, 1996 #39], [Ahmed, 1997 #40], we further investigated the influence of p53 function in EGR-1 mediated apoptosis using normal MEF cells isogenic for *Egr*-1 status. The findings of this study indicated that *Egr*-1$^{-/-}$ MEF cells were highly radio-resistant than *Egr*-1$^{+/-}$ MEF cells. Resistance to radiation in *Egr*-1$^{-/-}$ MEF cells was attributed to degradation of radiation-induced p53 protein. Together, in this normal background, we found that the radiation induced proapoptotic function of EGR-1 is directly mediated by the target genes p53 and Rb [Das, 2001 #61]. Altogether, these studies indicate that EGR-1 plays an important role in the regulation of radiation-induced apoptosis through p53, Rb and TNF-α target genes.

In this study, we further investigated the function of *Egr*-1 in radiation-induced apoptosis using a mutant p53 prostate cancer cell line DU145. We demonstrate here that overexpression of EGR-1 protein directly transactivates Bax causing an imbalance in Bcl-2:Bax protein ratio and rendering enhanced susceptibility to radiation-induced apoptosis.

MATERIALS AND METHODS

Plasmid Constructs. The plasmid CMV-EGR-1, which encodes full-length EGR-1 protein, contains EGR-1 cDNA downstream of the CMV promoter in the vector pCB6+ [Madden, 1991 #22]. Plasmid pCMV-WT1-EGR-1, which encodes a dominant-negative mutant of EGR-1, contains a WT1-EGR-1 chimera downstream of the cytomegalovirus (CMV) promoter in vector pCB6+ [Madden, 1991 #22]. The reporter construct, EBS-CAT, contains three EGR-1 binding sites (CGCCCCGC) placed in tandem upstream of a minimal c-fos promoter and CAT cDNA. The control pCAT reporter vector was purchased from Promega. The full length Bax promoter region was generated from human genomic DNA template by PCR using the sense primer flanking nucleotide −959 to −937 (5' TTTTTCTAGACAGCACAGATTAGTTTCTGCCAC- 3') and the antisense primer sequences from −18 to −1 (5'- TTTTAAGCTTCACCGCCGCTCCCGCCGC-3') contained built-in-sites (underlined) for XbaI and Hind III, respectively. The Bax promoter without EGR-1 binding site was generated from human genomic DNA by PCR using the sense primer flanking nucleotide − 823 to − 807 (5'- TTTTTCTAGAATGCTTGAGTCTGGGAGTTCA -3') and the antisense primer sequences from −73 to −56 (5'- TTTTAAGCTTCCCGGGTCACGTGAGAGC-3') contained built-in-sites (underlined) for XbaI and Hind III, respectively. The full length Bax promoter (pBax-CAT) and minus EBS Bax promoter (pΔBax-CAT) was cloned in pG-CAT reporter plasmid.

Cell Culture. DU145 prostate cancer cells that has mutated p53 gene was originally obtained from American Type Culture Collection (Manassas, VA), were grown in α-MEM medium supplemented with 10% fetal bovine serum and 1% Penicillin-streptomycin at 37^0C and 5% CO_2. Pools of transfected DU145 clones, DU145/Vector containing the pCB6+ vector, DU 145/CMV-EGR-1 expressing the cDNA of *Egr*-1, or DU145/CMV-WT1-EGR1 expressing the dominant-

negative mutant chimera for EGR-1 were grown in supplemented medium with G418 sulfate (400 μγ/ml).

DNA Transfection and CAT Assays. Stable transfection were performed by Lipofectamine plus reagent (Gibco BRL). Transient transfections were performed by the calcium phosphate co-precipitation method as described previously (37). CAT assays were performed by thin-layer chromatography as described previously [Ahmed, 1997 #40].

Irradiation. A 100 kV industrial X-ray machine (Phillips, Netherlands) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm aluminum plus 1-mm beryllium filter was 2.64 Gy/min at a focus-surface distance of 10 cm.

Immunocytochemistry. EGR-1 expression was determined in untreated and irradiated DU145 cells by immunocytochemical analysis, by using anti-EGR-1 antibody, sc-110 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and described by as previously [Ahmed, 1996 #39].

Fluorescence in situ Hybridization (FISH) Analysis of Egr-1 Gene . To evaluate the copy number or amplification of the *Egr*-1 gene, FISH was performed using a spectrum orange-labeled *Egr*-1 probe (locus: Egr-1 band assignment.: 5q23-31.1) obtained from Vysis Inc. (Downer Grove, IL) as describe previously [Ahmed, 1996 #39].

Quantitation of Apoptosis. Apoptosis was quantified by TUNEL staining and flow cytometry. The ApopTag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD), that detects DNA strand breaks by terminal transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) was used as described [Ahmed, 1997 #40]. Briefly, cells were seeded in chamber slides and the next day they were exposed to a 5Gy dose of radiation. After 24 h, the DNA was tailed with digoxigenin-dUTP and conjugated with an anti-digoxigenin fluorescein. The specimen was counter stained with propidium iodide and antifade. The stained specimen was observed in triple band –pass filter using Nikon-microphot epifluorescence microscope. To determine the percentage of cells

showing apoptosis, four experiments in total were performed, and approximately 1000 cells were counted in each experiment. For flow cytometry, cells were lifted by using non-enzymatic cell dissociation medium (Sigma) and washed with phosphate-buffered saline and stained with Hoechst (Ho342) and merocyanine (MC540) and analyzed by flow cytometry using a FACStar Plus cell sorter as described [Ahmed, 1997 #40].

Bax mRNA expression level by [32]P- Reverse Transcriptase-Polymerase Chain Reaction (^{32}P-RT-PCR). Total RNA was isolated from untreated and irradiated DU145 transfectant cells at various time intervals using TRIzol reagent (Life Technologies). One μg of total RNA was reverse transcribed into cDNA using oligo (dT) primers and reverse transcriptase in a 20-μl reaction mix as described previously [Ahmed, 1997 #40]. Radiation induced mRNA expression of Bax was analyzed by ^{32}P-RT-PCR. PCR was performed by using the products of reverse transcription reaction and the 5'-end radiolabeled upstream primer (5'-CCAGCTCTGAGCAGATCATGAAG-3') corresponding to nucleotide 41-63 and downstream primer (5'-GCAATCATCCTCTGCAGCTCCAT-3') corresponding to 220-242 of the Bax gene [Oltvai, 1993 #90]. Par-4 mRNA expression was performed by ^{32}P-RT-PCR. PCR was performed by using the products of reverse transcription reaction and the 5'-end radiolabeled upstream primer (5'-GATATAACAGGGATGCAA-3') corresponding to nucleotide 996-1013 and downstream primer (5'-AATGTATTGCAGCATAGG-3') corresponding to 1325-1344 of the death domain in Par-4 gene [Johnstone, 1996 #92]. In addition, G6PDH gene (the sense primer starts at nucleotide 1900, 5'-CCAAGCCCATCCCCTATA-3', and the antisense primer begins at nucleotide 1971, 5'-GGTGCCCTCATACTGGAA-3' and generates a 89 bp PCR product) was used as an internal control.

Western Blot Analysis. Total protein extracts from untreated and irradiated cells at various time intervals were subjected to Western blot analysis as described [Ahmed, 1996 #39], using anti-

EGR-1 antibody (sc-110) (Santa Cruz, CA), Bax antibody (Santa Cruz,CA; sc-7480), Bcl-2 (Santa Cruz, CA; sc-509), Caspase 3 (BioVision, CA; cat # 3004-100), PARP antibody (BioVision, CA; cat # 3001-100) or for loading control the anti-β-actin antibody (Sigma Chemical Co). The bound immune-complexes were detected using Chemiluminescence method (Santa Cruz, CA).

Colorimetric Assay for Caspase 3 and Caspase 9 Activities. Caspase 3 and Capase 9 activities were assayed colorimetrically using DEVD-pNA and LEHD-pNA substrates respectively, as per manufacturers recommendation (BioVision, CA). Briefly, untreated and irradiated DU145 transfected cells were resuspended in 50 µl of chilled Cell Lysis Buffer (BioVision, CA) and incubated on ice for 10mins. The suspension was centrifuged for a min at 10,000g and supernatant was collected as cytosolic protein extract. The protein content was measured by Bradford method [Bradford, 1976 #62]. Five hundred mg of the cytosolic protein extract in 50 µl was incubated with 50 µl of 2X Reaction buffer (containing 10mM DTT) and 5 µl of 4mM LEHD-pNA (for Caspase –9) or DEVD-pNA (for Caspase-3) for 1hr at 37°C. Then the activity was measured at 405 nm in a microtiter plate reader.

Electrophoretic Mobility Shift Assay. Nuclear extract were isolated from untreated and irradiated DU145 transfectants. Briefly, the cells were scraped and washed with cold PBS. Then the cells were suspended in 1 ml of ice cold Buffer A (10mM HEPES, pH 7.9, 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT, 0.5mM PMSF, 1µg/ml Leupeptin, and 0.1% Noniodet P 40) and incubated on ice for 20 min. The crude nuclei were pelleted by centrifugation for 5 min. The pellet was then suspended in 50µl of Buffer B (20mM HEPES, pH 7.8, 25% glycerol, 520 mM NaCl, 1.5mM MgCl₂, 0.1mM EDTA, 0.5mM DTT, 0.5mM PMSF, 1µg/ml Leupeptin, and 0.2 % Noniodet P 40). After 60 min incubation on ice, the suspensions were centrifuged for 20min at 4°C and supernatants were collected as nuclear extract. The protein content was measured by Bradford method [Bradford, 1976 #62].

Electrophoretic mobility shift assay was performed using Nushift kit for Egr-1 (Geneka Biotechnology, Montreal, Canada). The oligo consensus probe that contains Egr-1 binding site (underlined) is 5'-GGATCCAGCGGGGGCGAGCGGGGGCGAACG-3', and oligo consensus probe that contains the mutant Egr-1 binding site (underlined and mutation in small letter) is 5'-GGATCCAGCGGGGtaGAGCGGGtaCGAACG-3'obtained from Geneka Biotechnology (Montreal, Canada). The oligo consensus probe that has two overlapping potential Egr-1 binding sites (underlined) in Bax promoter is 5'-CGAGAGGCGGCGGCGGGAGCGGCGGTG-3'. Two mutant Bax promoters oligo were also synthesized : 5'-CGAGAGtaGGGattatcattaGGCGGTG3' and 5'-CGAGAGtttgcgtttggatttGCGGTG-3'. For super-shift experiments, rabbit EGR-1 polyclonal antibody (From Geneka Biotechnology, Montreal, Canada) was incubated with binding buffer and nuclear extract for 20 min at 10°C prior to adding oligo probe. Binding reactions were electrophoresed on a 5% polyacrylamide gel in 1x Tris-Glycine electrophoresis buffer to separate the bound and unbound probe.

RESULTS

DU 145 cells have three alleles for Egr-1 with low transactivation ability.

Almost all the prostate cancer cell lines exhibit complex chromosomal aberration. Based on this, analysis was performed to determine whether DU 145 cells have intact Egr-1 gene and was further determined the radiation-induction and transactivation potential of endogenous Egr-1. To achieve this, we first performed FISH to ascertain that Egr-1 alleles were intact in DU 145 cells. Egr-1 is mapped to the long arm of chromosome 5 (5q23-31.1). FISH analysis performed by using a spectrum orange-labeled Egr-1 probe, showed that in DU 145 cells the proportion of nuclei with more than three signals for the Egr-1 gene was 90-95% and that the proportion of nuclei with more than three signals was 5% to 10% (Figure 1A). Thus, FISH analysis confirmed the presence of three intact alleles of Egr-1 in DU 145 cells. DNA sequence analysis of Egr-1 gene in DU 145 cells suggested no presence of mutation.

To determine whether EGR-1 is also inducible to radiation in DU 145 cells, the cells were exposed to 5 Gy dose of radiation and total proteins were analyzed by Western blot analysis at different time points after radiation exposure for EGR-1 protein expression levels. Western blot analysis confirmed that DU 145 cells constitutively express modest levels of EGR-1 protein (Figure. 1B). After exposure to 5 Gy dose of radiation, EGR-1 protein showed slight induction at 15-30 minutes after radiation exposure (Figure 1B). Immunocytochemical analysis demonstrated an increase in nuclear EGR-1 expression levels after 1h of exposure to radiation (Figure 1C). The nuclear localization of EGR-1 is important particularly for transcriptional activation function of EGR-1 protein.

To ascertain the transcriptional potential of endogenous EGR-1 protein in untreated and irradiated DU 145 cells, we performed transient transfections using a reporter construct EBS-CAT, that contains three tandem EGR-1 binding sites placed in the upstream of minimal c-*fos* promoter

and CAT cDNA., and an EGR-1 expression construct CMV-EGR-1. In addition, as a control for CAT assays, we used the promoter of the *gro* gene placed upstream to CAT cDNA. This gene is strongly induced by IL-1 [Joshi-Barve, 1993 #74]. Thus, the gro–CAT was also transfected transiently in DU 145 cells to ascertain efficient functioning of the reporter gene CAT. As observed in Figure 1D, after 5 Gy dose of radiation, weak induction of CAT reporter activity (1.5%) was detected in DU 145 cells. CAT reporter activity was significantly induced when cells were co-transfected with CMV-EGR-1. Interestingly, a strong induction of CAT activity was also evident after radiation exposure when DU 145 cells were transfected with CMV-EGR-1 and EBS-CAT (Figure 1D). These findings suggest that radiation induced transactivation of EBS-CAT is very low when compared to CMV-EGR-1 mediated transactivation, implying that the endogenous EGR-1 protein in DU 145 may have low transactivation function.

DU 145 cells are resistant to radiation-induced apoptosis and clonogenic inhibition.

To determine whether ionizing radiation causes apoptosis in DU 145, cells were irradiated at 5-Gy dose and subjected to TUNEL after 24 h (Figure 2A). The TUNEL analysis indicated that radiation caused a marginal increase of apoptosis (4.5%) in DU 145 cells. The response of DU 145 cells to radiation was also assessed by colony-forming assays for clonogenic inhibition. In colony-forming assays, the SF_2 value of exponentially growing irradiated DU 145 cells was 0.54 with a D_0 value of 293 cGy and the n value was 1.001 (Figure 2B). Together these data suggest that DU 145 cells are resistant to radiation-induced apoptosis.

Stable overexpression of EGR-1 in DU 145 cells led to restoration of the transcriptional activity of EGR-1.

As shown in figure 1C, the endogenous EGR-1 in DU 145 cells failed to transactivate the EBS-CAT reporter construct after radiation, suggesting that the endogenous Egr-1 is defective in it's transcriptional activity. To restore the transcriptional activity of Egr-1 in DU 145 cells, we

ectopically overexpressed EGR-1 protein and further analyzed for transactivation potential of ectopically expressed EGR-1 using gel-shift and EBS-CAT reporter assays. We stably transfected the DU 145 cells with CMV-EGR-1 and dominant-negative mutant of EGR-1 (CMV-WT1-EGR-1) and with an empty vector as a control. We then selected Geniticin-resistant stable transfectant clones. Three clones from each transfectant group were analyzed for EGR-1 expression by Western blot analysis. Figure 3A shows that DU 145/CMV-EGR-1 showed appreciable high levels of EGR-1 protein when compared to DU 145/Vector or DU 145/CMV-WT1-EGR-1.

To ascertain the transactivation activity of ectopically expressed EGR-1 in DU 145 transfectants, electrophoretic mobility shift assays were performed. Nuclear protein extracts prepared from cells stably transfected with CMV/EGR-1 or CMV-WT-1/EGR-1 or vector alone were incubated with radiolabeled EBS containing double stranded oligo probe (Geneka Biotechnology, Montreal Canada). A subtle increase in EGR-1 binding activity was observed in irradiated DU 145/Vector transfectants when compared to untreated cells. Whereas, in DU 145/CMV-EGR-1 cells, a significant increase in EGR-1 binding activity was observed both in untreated and irradiated nuclear extracts. In DU 145/CMV-WT1-EGR1 cells, no EGR-1 binding activity was evident in both untreated and irradiated samples (Figure 3B). Next, the DU 145 transfectants were assessed for reporter CAT activity using EBS-CAT reporter construct. DU 145/Vector and DU 145/CMV-WT1-EGR1 transfectant cells showed low CAT activity and DU 145/CMV-EGR-1 transfectants showed a significant increase in the CAT activity (Figure 3C). These results indicate that stable expression of EGR-1 restored the transactivation potential of EGR-1 target sequence in DU 145/CMV-EGR-1 cells.

Ectopic expression of EGR-1 rendered enhanced sensitivity to radiation-induced clonogenic inhibition and apoptosis.

Having demonstrated that ectopically expressed EGR-1 restored the EGR-1 binding activity in DU 145 cells, we determined its impact on radiation response. Initially, we determined the effect of abrogation of endogenous EGR-1 function in DU 145 cells expressing the chimera in response to ionizing radiation. In colony-forming assays, the SF_2 value of exponentially growing irradiated DU 145/Vector cells was 0.609 with a D_0 value of 400 cGy (Figure. 4A). On the other hand, the SF_2 for DU 145/CMV-WT1-EGR1 was 0.66 with the D_0 value of 509 cGy, suggesting that compared to vector-transfected cells, the cells transfected with the chimera were more resistant ($p<0.001$) to ionizing radiation. Then, we tested the effect of ectopic EGR-1 overexpression in DU 145 cells stably transfected with CMV-EGR-1 on the response to ionizing radiation. As seen in Figure 4A, the SF_2 for DU 145/CMV-EGR1 was 0.34 with the D_0 value of 164 cGy, suggesting enhanced sensitivity ($p<0.0001$) than the vector transfected cells to ionizing radiation. Consistent with this observation, flow analysis and TUNEL assay indicated that DU 145/CMV-WT1-EGR1 cells were resistant ($p<0.001$) and DU 145/CMV-EGR-1 cells were significantly more sensitive ($p<0.0001$) to ionizing radiation-inducible apoptosis than vector transfected DU 145 control cells (Figure 4B and 4C). Together these findings suggest that EGR-1 is required for ionizing radiation-inducible apoptosis and that when overexpressed EGR-1 potentiates the effects of ionizing radiation.

DU 145/CMV-EGR-1 transfectant showed high Bax levels and inhibited radiation-induced Bcl-2 protein expression.

To further elucidate the mechanism of radiation sensitization by ectopic expression of EGR-1 in DU 145 cells, we hypothesized that the pro-apoptotic effector proteins and downstream apoptotic effector proteins might have been altered to confer enhanced radiation sensitivity. Western blot analysis of DU 145 transfectants indicated a significant upregulation of Bax protein in both untreated and irradiated DU 145/CMV-EGR-1 cells when compared to DU 145/CMV-WT1-EGR1 or vector transfected control. Interestingly, DU 145/CMV-EGR-1 transfectants also showed downregulation of Bcl-2 protein in response to radiation when compared to elevated levels of Bcl-2 protein expression in irradiated DU 145 cells transfected with chimera or vector alone (Figure 5A).

The up-regulation of Bax is believed to effect mitochondria permeability, favoring the release of cytochrome C, leading to Caspase-9 activation, which subsequently induces Caspase-3 activation [Tsujimoto, 1998 #80]. Since DU 145/CMV-EGR-1 transfectant cells showed an increase in the level of Bax protein, we further characterized the downstream events of cell death by analyzing the activated form of Caspase-3 and PARP cleavage by Western blot analysis. In DU 145/Vector and DU 145/CMV-WT1-EGR1 transfected cells, radiation caused the elevation of inactive forms of Caspase-3, with no PARP cleavage (Figure 5B), whereas, in DU 145/CMV-EGR-1 cells, radiation caused the activation of Caspase-3 by reducing the inactive forms of Caspase-3 and was associated with enhanced cleavage of 116-kDa PARP (Figure 5B). Since Caspase-3 was cleaved in DU 145/CMV-EGR-1 cells, we assessed the activity of Caspase-9 and Caspase-3 in untreated and irradiated DU 145 transfectants. After radiation, a significant induction of Caspase-9 (Figure 5C) and Caspase-3 activity (Figure 5D) was observed when compared to vector or chimera transfected DU 145 cells. These observations indicate that EGR-1 is a positive effector of radiation-induced apoptosis through Bax and Caspase activation.

EGR-1 binds to GC-rich sites of Bax promoter and upregulates Bax transcription.

Since Bax protein was significantly induced in DU 145/CMV-EGR-1, we hypothesized that the Bax promoter may have potential Egr-1 binding sites. Promoter sequence analysis of Bax gene showed two potential overlapping Egr-1 binding site flanking from –21 to –13 (GCGGCGGCG) and –15 to –7 (GCGGGAGCG). When compared to normal consensus sequence of Egr-1 binding site, GCGGGGGCG, both the overlapping Egr-1 binding sequence in the Bax promoter has one base variation in the center of the consensus sequence (Figure 6A). These sites are present just after CACGTG motifs which are potential binding sites for transcription factors that includes Myc and its homologs [Blackwood, 1991 #81]; [Zervos, 1993 #82]; [Ayer, 1993 #83]. To ascertain whether these two overlapping binding sequence form potential binding consensus for EGR-1 protein, we

performed gel-shift and CAT-reporter assays. Gel shift was performed by designing double stranded oligo probe spanning the two overlapping EBS consensus sequence in the promoter of Bax. A mutant double oligo probe was also synthesized by mutating the EBS sequence. For CAT-reporter assays, full-length Bax promoter containing both overlapping EBS sequence and minus EBS sequence were amplified and cloned into CAT reporter plasmid (Figure 6A). Gel shift analysis indicated that DU 145/CMV-EGR-1 cells showed enhanced binding to wild-type double-stranded oligo probe but not to the mutant probe (Figure 6B). The bound complex in DU 145/CMV-EGR-1 cells further shifted above when incubated with anti-EGR-1 antibody, confirming that EGR-1 protein binds to EBS consensus sequence in Bax promoter (Figure 6B). In the DU 145/Vector transfected cells, no band shift or super-shift was evident suggesting that endogenous EGR-1 is not functional. (Figure 6B).

We performed CAT assays by using a reporter constructs, pBax-CAT that contains the Bax promoter region from −959 to −1 and ΔBax-CAT that contains the Bax promoter region with no Egr-1 binding site sequence from −823 to -56. DU 145 cells were transiently cotransfected with pBax-CAT or ΔBax-CAT, CMV-EGR-1 and either the dominant negative mutant CMV-WT1-EGR-1 or the empty vector pCB6^{+}. Cells cotransfected with pBax-CAT and vector showed a modest background level CAT activity (Figure 6C). The CAT activity was increased significantly when the cells were cotransfected with pBax-CAT, CMV-EGR-1 and vector. On the other hand, when co-transfection was performed with pBax-CAT, CMV-EGR-1 and CMV-WT1-EGR-1, CAT activity was severely reduced (Figure 6C). These findings indicated that CMV-EGR-1 can transactivate, and the dominant-negative mutant WT1-EGR-1 can transrepress, the Bax promoter. When a deletion, that abolished the ability of EGR-1 protein to bind to the Bax promoter, was introduced into the pBax-CAT construct, basal expression driven by the promoter was attenuated

(Figure 6C). As expected, ectopically expressed EGR-1 caused induction of CAT activity from the promoter construct that contained wild-type EGR-1-binding site but not from the construct that contained the deleted EGR-1-binding site (Figure 6C). Together, these findings suggest that EGR-1 transactivates the Bax promoter via the EGR-1-binding site and that this site is required for Bax upregulation.

Since EGR-1 directly binds to Bax promoter sequence, we further evaluated the effects of overexpression of EGR-1 protein on the Bax mRNA expression. RT-PCR analysis indicated significant elevated levels of Bax mRNA in untreated and irradiated DU 145/CMV-EGR-1 transfectants when compared to vector control or chimera transfected DU 145 cells (Figure 6D). Thus, these observations strongly indicate that Bax is target gene for Egr-1 in radiation-inducible apoptosis.

DU 145/CMV-EGR-1 transfectant showed elevation of pro-apoptotic gene Par-4 and inhibition of NFκB activation.

Bcl-2 expression is regulated by two important transcription factors, p53 and NFκB, both of which have opposite roles under cellular insults. The tumor suppressor gene, p53, is a potent transcriptional repressor of Bcl-2 [Mollenkamp, 1975 #68], whereas, NFκB is potent transcriptional activator of Bcl-2 [Tamatani, 1999 #84]; [Dixon, 1997 #78]. On cellular insults, p53 represses Bcl-2 [Miyashita, 1994 #76] and simultaneously Bax is induced by p53 [Miyashita, 1995 #46] causing a change in the bcl-2: bax ratio leading to the culmination of downstream cell-death processes. On the contrary, NFκB mediated induction of Bcl-2 protein will lower the Bax ratio leading to enhanced cell survival. Many studies have shown that ionizing radiation decreases the Bcl-2 protein levels in p53 wild-type cell lines causing enhanced cell death [Chen, 1995 #79][Zhan, 1999 #85]. On the other hand, in cell lines' lacking wild-type p53 protein radiation was found to

upregulate Bcl-2 [Kariya, 1999 #86]. Loss of p53 function and radiation-induced Bcl-2 upregulation may contribute towards enhanced resistance to apoptosis [Kariya, 1999 #86]. In DU 145 cells transfected with vector alone or chimera, radiation caused an induction of Bcl-2 protein, whereas, in DU-145/CMV-EGR-1 cells, radiation caused down-regulation of Bcl-2 protein. Previously, it has been reported that Egr-1 represses Bcl-2 [Huang, 1998 #87] and was also found to inhibit the NFκB activity by directly binding it to RHD region of Rel A (p65) [Chapman, 2000 #54]. It has been shown that atypical PKCs specifically bind to pro-apoptotic gene Par-4, which is exclusively induced on death stimuli [Sells, 1994 #88], causing inhibition of cell proliferation and cell survival in cells undergoing apoptosis [Diaz-Meco, 1996 #89]. More interestingly, Par-4 was found to inhibit TNF-α induced nuclear translocation of p65 as well as κB-dependent promoter activity through inhibition of IκB phosphorylation by directly blocking the inhibitory κB protein (IκB) kinase activity [Diaz-Meco, 1996 #89]. To further understand the mechanism of Egr-1 mediated radiation-induced apoptosis, we analyzed the mRNA and protein of Par-4 and κB activity. RT-PCR analysis showed increased Par-4 mRNA expression in untreated and irradiated DU 145/CMV-EGR-1 cells with increased Par-4 protein expression (Figure 7A and 7B). However, in vector transfected cells, Par-4 mRNA was elevated with protein levels unaltered and in CMV-WT1-EGR-1 transfected DU 145 cells, Par-4 mRNA and protein levels were down-regulated after radiation (Figure 7A and 7B). Interestingly, the kB-CAT activity was induced in vector transfected cell after radiation and co-transfection with CMV-EGR-1 reduced the radiation-induced kB-CAT activity suggesting that EGR-1 can directly inhibit radiation-induced NFkB activity (Figure 7C). Together, these findings suggest that EGR-1 mediated radiation-induced apoptosis is signaled through inhibition of radiation-induced pro-survival factors such as Bcl-2 and NFkB activity and upregulation of pro-apoptotic genes such as Bax and Par-4, culminating into activation caspases and cell death program (see pathway, Figure 8).
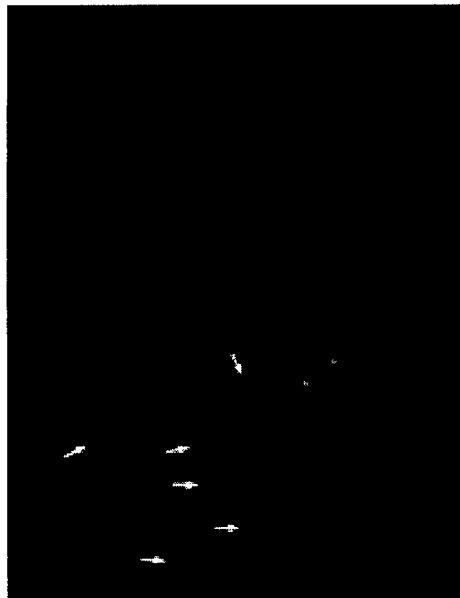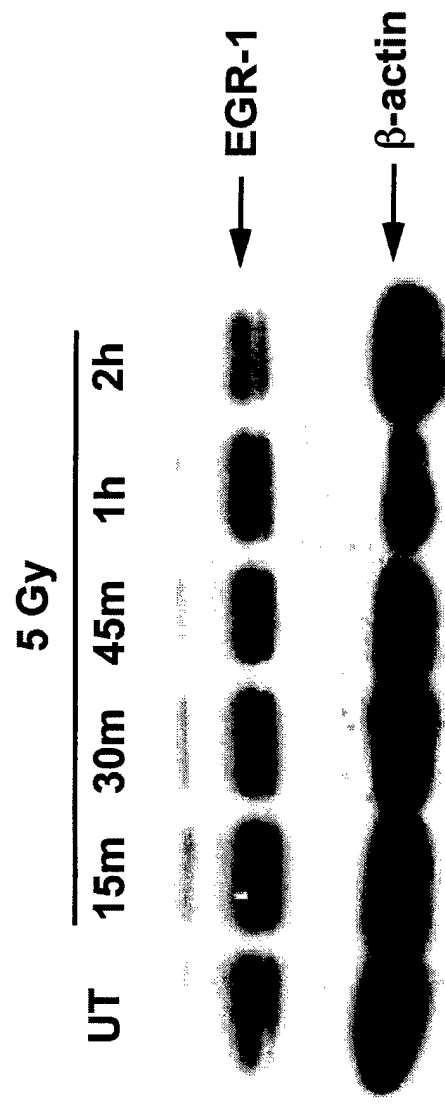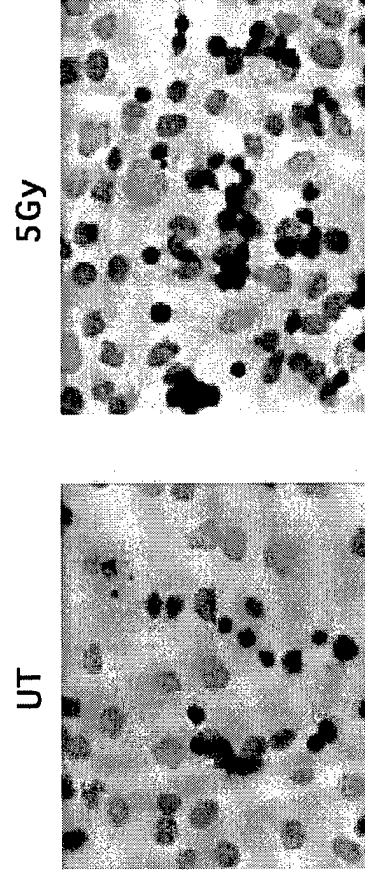
Das et al

Figure 1A

Das et al

Figure 1B

5 Gy

UT 15m 30m 45m 1h 2h

EGR-1

β-actin

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UT   5Gy

Figure 1C

Das et al

Figure 1D

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Figure 2A

Figure 2A. Bar graph showing PERCENT TUNEL POSITIVE CELLS for DU-145 under Untreated and 5 Gy (24 hours) conditions, with y-axis from 0 to 10.

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Figure 2B

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EGR-1

β-actin

1   2   3

1. DU-145 / Vector
2. DU-145 / WT1-EGR-1
3. DU-145 / EGR-1

Figure 3A

Figure 3B

Das et al

1. DU-145/Vector + EBS oligo probe
2. DU-145/Vector + EBS oligo probe + radiation
3. DU-145/CMV-EGR1 + EBS oligo probe
4. DU-145/CMV-EGR1 + EBS oligo probe + radiation
5. DU-145/CMV-WT-EGR1 + EBS oligo probe
6. DU-145/CMV-WT-EGR1 + EBS oligo probe + radiation
7. DU-145/CMV-EGR1 + EBS oligo probe + EGR-1 antibody
8. DU-145/CMV-EGR1 + EBS oligo probe + EBS cold oligo
9. EBS oligo probe only

SUPER SHIFT
EGR-1 BAND SHIFT
Non-specific 1
Non-specific 2
Free probe

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Figure 3C

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Figure 4A

■ DU-145/Vector ● DU-145/CMV-WT1-EGR1 ▲ DU-145/CMV-EGR1

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Figure 4B

Das et al

Figure 4C

Das et al

Figure 5A

Das et al

Figure 5B

Das et al

Figure 5C

Das et al

Figure 5D

Das et al

Figure 6A

pBax-CAT

CAT

p53 binding site
TATAA box
Transcription start site
-959

CACGTG motif
-1

EGR-1 BINDING SITES
5'-CGAGACGCGGGGCGGGGAGCCGGGCGGGTG-3'
EBS-1
EBS-2
-21
-7

pΔ Bax-CAT

CAT

p53 binding site
TATAA box
Transcription start site
-823

CACGTG motif
-56

Das et al

Figure 6B

1. DU 145/Vector + Bax oligo probe
2. DU 145/Vector + EBS oligo probe + EGR-1 antibody
3. DU 145/Vector+ Bax oligo probe + radiation
4. DU 145/CMV-EGR-1 + Bax oligo probe
5. DU 145/CMV-EGR-1+ Bax oligo probe + radiation
6. DU 145/CMV-EGR-1 + Bax oligo probe + EGR-1 antibody
7. DU 145/CMV-EGR-1 + Bax oligo probe + cold Bax oligo
8. DU 145/CMV-EGR-1 + Mutant 1 Bax oligo probe
9. DU 145/CMV-EGR-1 + Mutant 2 Bax oligo probe
10. Bax oligo probe only

Non-specific band
Specific band shift
Non-specific bands
Free probe

Das et al

Figure 6C

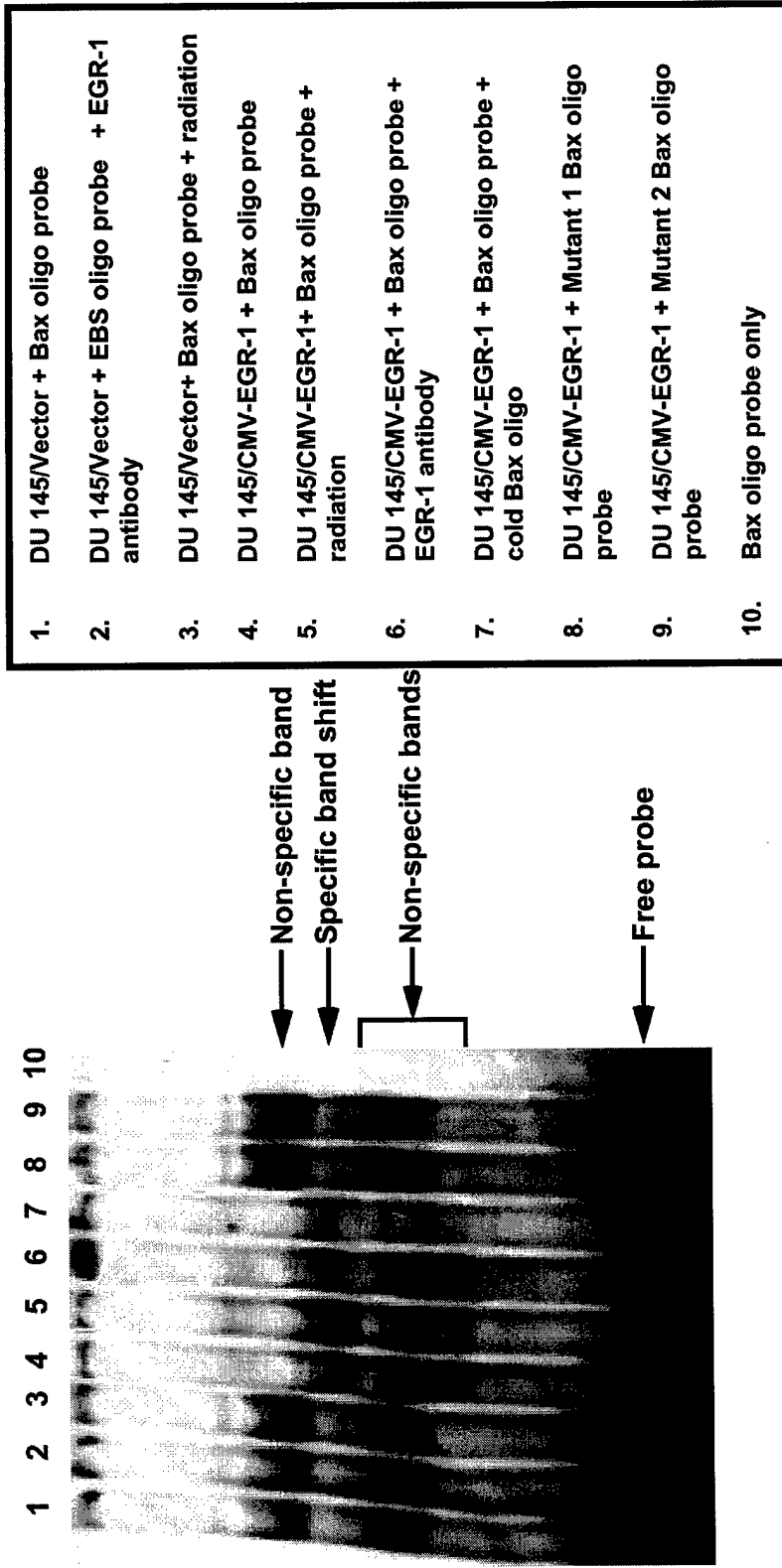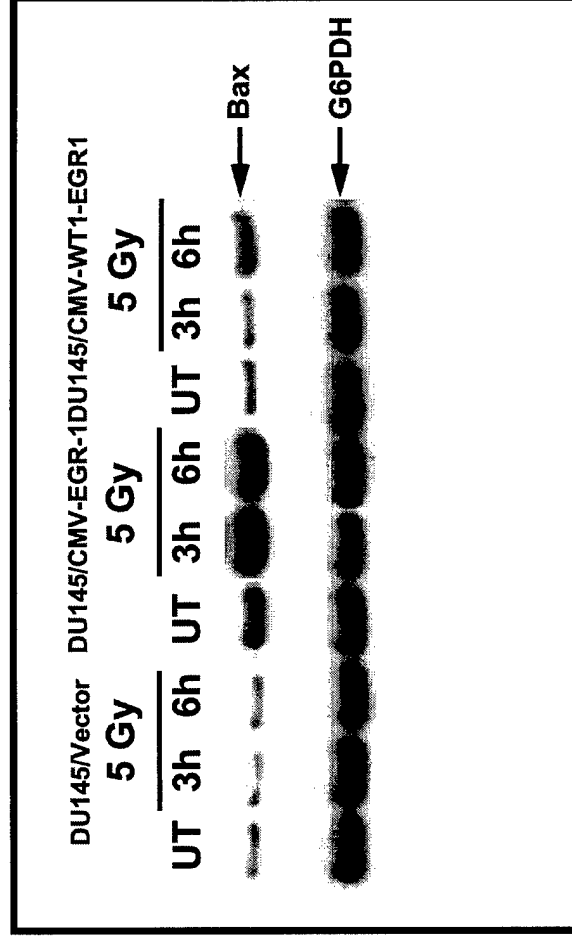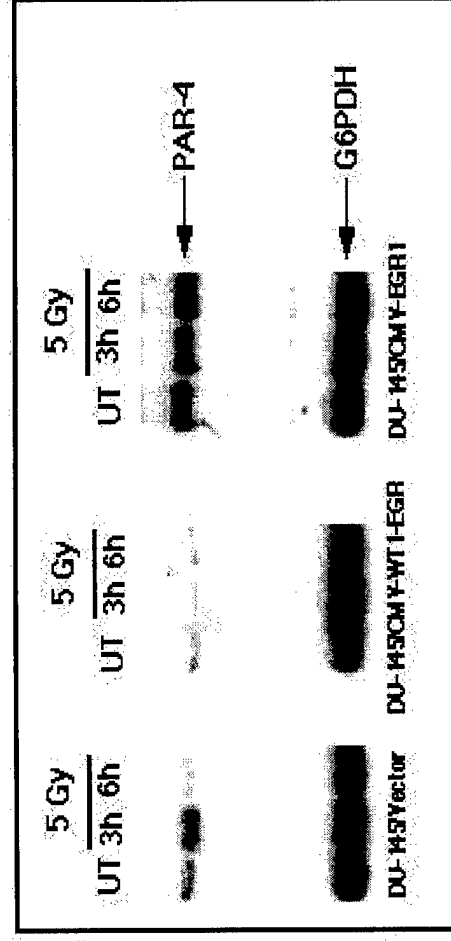|	pCAT	Empty Vector	pBax-CAT	pΔ Bax-CAT	CMV-EGR-1	CMV-WT1-EGR-1	EBS-CAT	Percent conversion
	+	-	-	-	-	-	-	65.2%
	-	+	+	-	-	-	-	13.8
	-	+	-	+	-	-	-	17.5%
	-	-	+	-	+	-	-	58.9%
	-	-	-	+	+	-	-	12.2%
	-	-	+	-	-	+	-	1.2%
	-	-	-	+	-	+	-	1.8%
	-	-	+	-	+	+	-	33.2%
	-	-	-	+	-	+	-	1.9%
	-	+	-	-	-	-	+	6.9%
	-	-	-	-	+	-	+	28.7%

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Figure 6D

Das et al

Figure 7A

Das et al

Figure 7B

Figure 7C

Figure 8

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ASSISTANCE AGREEMENT

AWARD TYPE: ☑ GRANT (31 USC 6304) ☐ OPERATIVE AGREEMENT (31 USC 6305) ☐ ER TRANSACTION (10 USC 2371)

AWARD NO: DAMD17-98-1-8473 Modification P90004	EFFECTIVE DATE See Grants Officer Signature Date Below	AWARD AMOUNT $229,187.00	Page 1 of 19 Shannyn M. Scassero Phone 301-619-2640 Fax 301-619-2505

PROJECT TITLE: Role of Early Growth Response-1 (Egr-1) Gene in Radiation-Induced Apoptosis of Prostate Cancer Cells
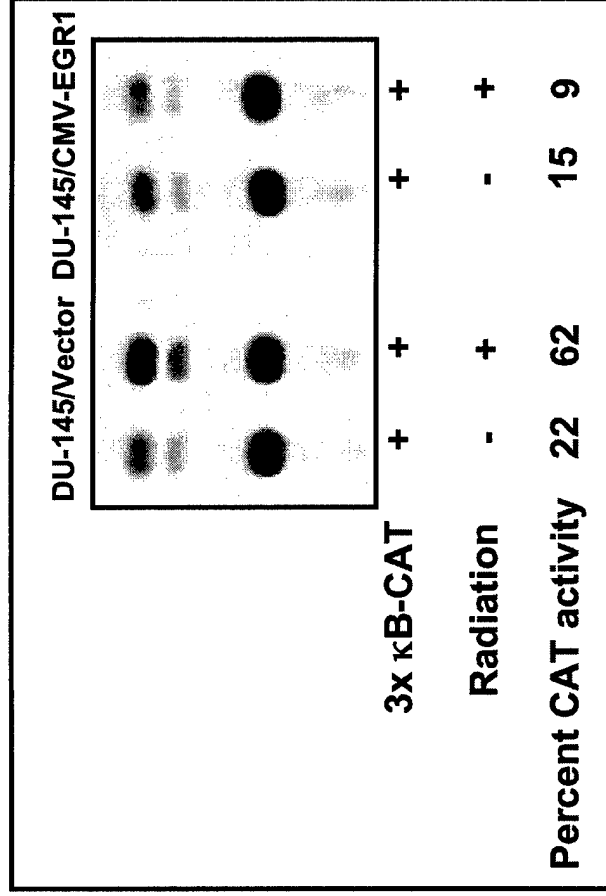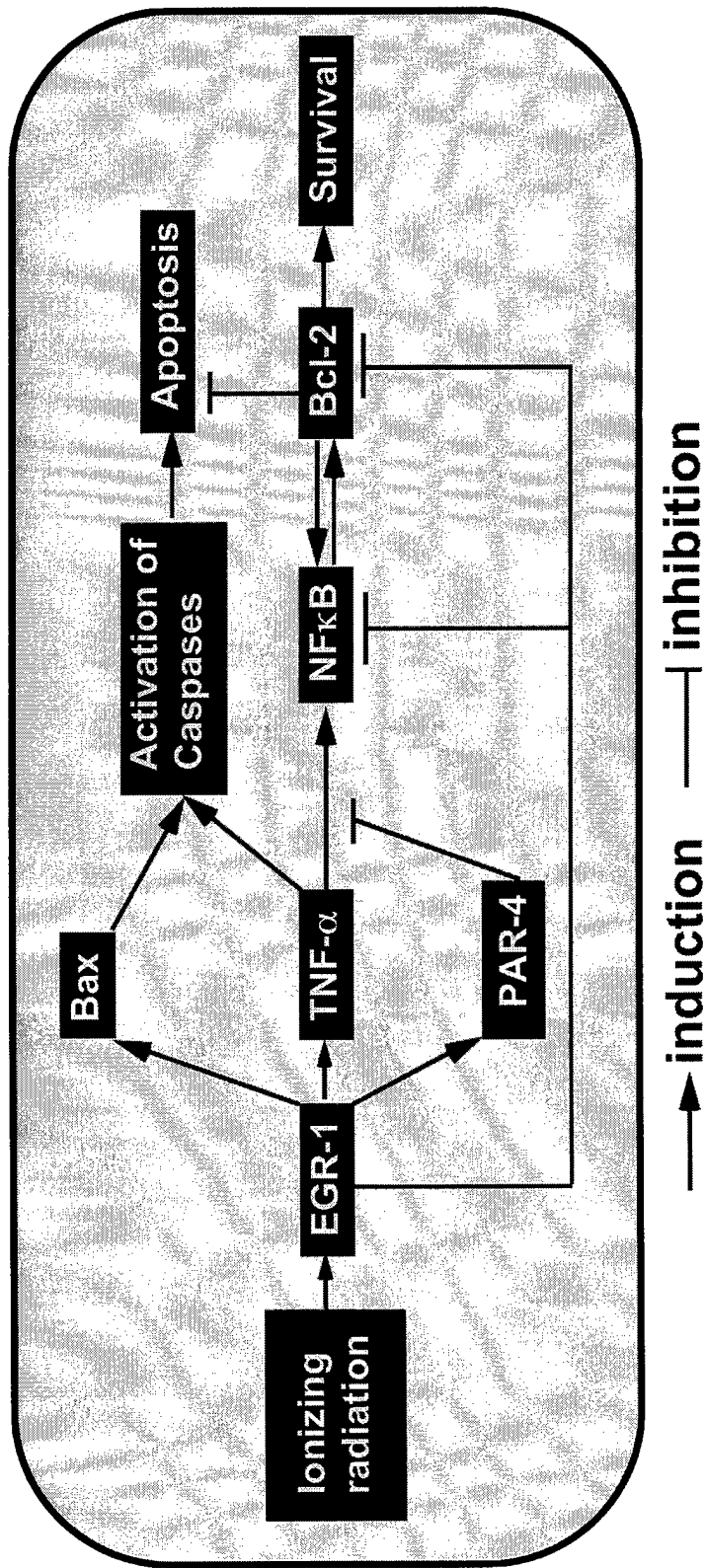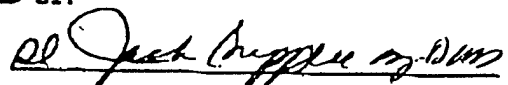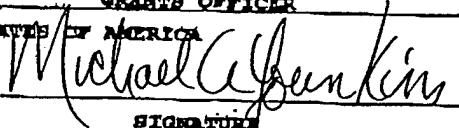
CFDA 12.420

PERFORMANCE PERIOD: 1 AUG 98 - 28 FEB 01 (Research to end 31 JAN 01)	PRINCIPAL INVESTIGATOR: Mansoor M. Ahmed, Ph.D.
AWARDED AND ADMINISTERED BY: U.S. Army Medical Research Acquisition Activity ATTN: MCMR-AAA-V 820 Chandler St. Fort Detrick Maryland 21702-5014	PAYMENTS WILL BE MADE BY: EFT:T Army Vendor Pay DFAS-SA/PPA 500 McCullough Avenue San Antonio, TX 78215-2100
AWARDED TO: University of Kentucky Research Foundation 2091 Kinkead Hall Lexington, KY 40506-0087	REMIT PAYMENT TO: University of Kentucky Research Foundation c/o Manager Office of Sponsored Projects Accounting 337 Peterson Service Building Lexington, KY 40506-0005

ACCOUNTING AND APPROPRIATION DATA:
2182040000087481196227879209Y415000FEMH0DAMD179818473FEMH00S18064   (-$268.00)

SCOPE OF WORK:

A. The purpose of this modification is to delete the technical proposal, dated 20 NOV 97 and budget dated 30 JUN 98. The attached Statement of Work dated 8 JUN 99 and budget dated 22 JUN 99 are incorporated herein by reference. The University of Kentucky requested this change, in a letter dated 7 MAY 99, to alleviate the problem of overlapping tasks with a National Institiute of Health grant. The revised budget is reduced by $268.00 from $229,455.00 to $229,187.00. The Grant Officer's Representative, Dr. Mishra, approved this change in a memorandum dated 18 JUN 99.

B. C. Paragraph 11, Payment Schedule is changed in the following manner:

C. The schedule of payments is as follows:

Year Two	$92,658 (-82)
Amount	On or About
$23,165	1 JUL 99
$23,165	1 OCT 99
$23,164	1 JAN 00
$23,164	1 APR 00

RECIPIENT	GRANTS OFFICER
ACCEPTED BY: _[signature]_ SIGNATURE	UNITED STATES OF AMERICA _[signature]_ SIGNATURE
NAME AND TITLE JACK SUPPLEE ASSOC. DIRECTOR	DATE 7/1/99	NAME AND TITLE MICHAEL A. YOUNKINS GRANTS OFFICER	DATE 7/2/99

USAMRAA FORM 60-R, Dec 98

UNIVERSITY OF KENTUCKY
RESEARCH FOUNDATION

PROPOSAL

SPECIFIC AIMS

Tumor aggressiveness correlates with enhanced resistance to apoptosis. Treatment strategies such as chemotherapy and radiation eliminate malignant cells by the induction of apoptosis as well as by "mitotic death". For example, therapeutic ionizing radiation can cause DNA strand breakage or distortion of the DNA nucleoprotein conformation which may transduce signals that result in activation of early response genes (*c-jun*, *c-fos* and *Egr*-1) whose gene products may then stimulate later genes such as TNF-α, pRB, IL-1 and PDGF-α. These later genes are important in cellular response to radiation injury (such as cell death). Therefore, it is to the advantage of tumor cells to acquire mutation and overexpression of these cellular genes which protect against these process.

Our previous studies in *p53* deficient prostate cancer cell line PC-3, suggest that overexpression of wild type EGR-1 protein markedly increased radiation-induced growth inhibition and apoptosis and this phenomenon was mediated by the induction of TNF-α (the promoter of TNF-α contains one *Egr*-1 binding site). However, overexpression of dominant-negative mutant protein targeted against the function of wild-type *Egr*-1 resulted in decreased radiation-induced clonogenic inhibition and apoptosis, and this mechanism caused significant down-regulation of TNF-α mRNA and protein expression. Our recent studies using mouse embryonic fibroblasts (MEFs) cells from *Egr*-1$^{-/-}$ and *Egr*-1$^{+/-}$ mice suggest that cells completely lacking *Egr*-1 were highly radioresistant when compared to cells containing one allele of *Egr*-1. Ionizing radiation caused elevation of *p53* protein in *Egr*-1$^{+/-}$ cells, however, in *Egr*-1$^{-/-}$ cells *p53* protein was downregulated. In addition, low levels of pRB protein (is a *Egr*-1 target gene and a central mediator of growth regulation after DNA damage) was found in *Egr*-1$^{-/-}$ MEF cells than in *Egr*-1$^{+/-}$ cells. Recently, it was reported that pRB regulates the stability of p53 via MDM-2. Thus, degradation of p53 in irradiated *Egr*-1$^{-/-}$ MEF cells may relate to low levels of pRB protein present in *Egr*-1$^{-/-}$ MEFs. This observation underscores an important novel mechanism involving *Egr*-1 as a key upstream regulatory gene in the control of apoptosis process which is mediated through wild-type *p53* and pRB genes.

HYPOTHESIS/PURPOSE

Based on these observations, we hypothesize that in prostatic carcinoma cells radiation induces wild-type EGR-1 protein expression, leading to the upregulation of p53 and TNF-α protein resulting in apoptosis and cell death. The objectives proposed below will directly address these hypotheses. If our *in vitro* studies provide a functional link between EGR-1 expression and radio-resistance (mediated through TNF-α pathway and irrespective of *p53* status) in prostate cancer, we will plan experiments to ascertain that EGR-1 expression plays a protective role in an animal model for prostate cancer. A long-term goal of this project is to identify patients who carry tumor sub-populations with intrinsic radiation-resistance and who may benefit from selective radiation dose escalation. Finally, if this hypothesis is correct, this study will also suggests that androgen depletion (hormone) treatment should be combined with radiation therapy to enhance cell-killing response in prostatic tumors exhibiting wild-type functional *Egr*-1 gene.

TECHNICAL OBJECTIVES

1. To determine the induction of EGR-1 gene protein expression in established cultures of prostate cancer xenograft line CWR22R (an androgen-independent recurrent subline of CWR22 xenograft) exposed to radiation and characterize the radiosensitivity profile of these cells with respect to apoptotic rate, growth inhibition, and colony-forming ability. These results will be compared to the radiosensitivity profile of its androgen-dependent counterpart CWR22.

2. To determine the functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cell line CWR22R and the androgen-dependent CWR22. This will be performed in transfectant cell lines expressing the *Egr*-1 dominant-negative mutant or in parent cells where *Egr*-1 expression is blocked by *Egr*-1-antisense oligomers and also in transfectant cell lines overexpressing the cDNA of *Egr*-1.

3. To elucidate the pathway of *Egr*-1 mediated regulation of p53 and TNF-α genes so that the differential mechanism of radiation-inducible apoptosis in prostate cancer cells (CWR22R) and the androgen-dependent CWR22 cells can be understood. This will be performed by studying the radiation-induced kinetics of p53 and TNF-α mRNA and protein in transfectant cell lines expressing the *Egr*-1 dominant negative mutant protein or in transfectant cell lines overexpressing the cDNA of *Egr*-1 by ^{32}P-RT-PCR and Western blot analysis respectively. Also, in these transfectants, CAT and gel-shift assays will be performed using the promoter of TNF-α and p53 to understand the transactivation mechanism by which EGR-1 may upregulate p53 and TNF-α.

4. To understand the mechanism of radiation-induced apoptosis in isogenic mouse embryonic fibroblast (MEF) cells with homozygous (Egr-1$^{+/+}$), heterozygous (Egr-1$^{+/-}$) and null (Egr-1$^{-/-}$) allelic status for Egr-1

1

gene. This will be performed by studying the radiation-induced kinetics of target genes such as p53, TNF-α and pRB at the mRNA and protein level in MEF cells with Egr-1$^{-/-}$ and Egr-1$^{+/+}$ background. In addition, CAT assays will be performed using p53-CAT, TNF-CAT and pRB-CAT constructs to understand the Egr-1 mediated regulation of transcription of p53, TNF-α and pRB genes.

An analysis of the molecular indicators of cell proliferation and apoptosis may lead to identification and management of prostate cancer patients with inherent resistance to hormone or radiation therapy. The Radiation Therapy Oncology Group (RTOG) currently has four protocols open for studying androgen ablation combined with radiation therapy in the treatment of carcinoma of prostate. If this hypothesis is correct, this study will recommend to RTOG to identify patients exhibiting wild-type functional *Egr*-1 gene as candidates for androgen depletion by hormone treatment combined with radiation therapy to enhance cell-killing response in prostatic tumors.

2

Research Design & Methods

Specific aim #1: To determine the induction of EGR-1 gene protein expression in established cultures of prostate cancer xenograft line CWR22R (an androgen-independent recurrent subline of CWR22 xenograft) exposed to radiation and characterize the radiosensitivity profile of these cells with respect to apoptotic rate, growth inhibition, and colony-forming ability. These results will be compared to the radiosensitivity profile of its androgen-dependent counterpart CWR22.

Rationale: Induction of *Egr-1* by radiation is expected to activate transcription via the GC-rich DNA binding sites present in several promoters of downstream genes leading to alteration in their expression profile and a phenotypic response. A recent report suggests that the *Egr-1* gene is one of a growing set of tumor suppressor-type genes (1). In order to understand the role of *Egr-1* in growth control of prostate cancer cells, this project proposes to investigate the functional relevance of radiation-inducible transient induction of EGR-1 protein in clonogenic inhibition or cell death. Till to date, there are nine human prostate cancer cell lines and 21 xenograft tumors, derived from primary and metastatic human prostate tumors (2-4). Two xenograft tumors (CWR22 and CWR22R) will be used in this specific aim. CWR22 was derived from Gleason tumor grade 9, stage D prostate carcinoma with osseous metastasis. It is androgen-dependent, does not grow in female mice, and regresses in male mice after orchiectomy. CWR22R, a subline of CWR22 that recurred after regression upon androgen withdrawal, is not dependent on androgen and is able to grow in female and castrated animals. Thus CWR22 and CWR22R represent the first pair of human androgen-dependent and –independent xenograft tumors derived from the same patient tumor. These two xenograft cell lines will form excellent model to study the *Egr*-1 mediated regulation of radiation-inducible apoptosis in paired androgen-dependent and –independent prostate tumors.

Experimental design: Initially, clonogenic survival and growth inhibition profiles of these cell lines following exposure to different doses of radiation will be determined by colony-forming assay and [³H] thymidine-incorporation assay. Plating efficiencies will be determined by the colony-forming assay. For clonogenic assay, plates will be stained with crystal violet and colonies will be counted 10-14 days after irradiation. Only colonies containing >50 cells will be scored as representative of surviving cells. For [³H] thymidine-incorporation assay, cells will be plated at a density of 10,000/ml and irradiated after 24 h. After 48 h of culture, the cells will be pulsed for 8 h with [³H]thymidine (2 Ci/mmol; 0.5 µCi/well) and radioactivity incorporation will be determined.

We will also characterize all the two cell lines to determine whether these cells harbor wild or mutant *Egr*-1. PCR will be performed using specific primers which will be designed using *Egr*-1 genomic sequence and the products will be subsequently sequenced in both sense and antisense direction. Allelic status will be determined by flourescent *in-situ* hybridization (FISH). The genomic status of *Egr*-1 gene will be compared with the basal levels and radio-induction potential of *Egr*-1 gene. Basal levels of EGR-1 and radiation-inducible expression levels at different doses of radiation will be determined by immunocytochemistry. The kinetics of EGR-1 expression will be studied by Western blot analysis. For immunocytochemistry, irradiated cells will be incubated for 1 hr at 37°C and will be subjected to immunocytochemistry with the anti-EGR-1 antibody, sc-110 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For Western blot analysis, total cell extracts, obtained at various time intervals, will be electrophoresed on 7.5% polyacrylamide-SDS gels and transferred to nylon membranes. The membrane will be probed with EGR-1 antibody, sc-110 and incubated with ¹²⁵I protein A , as described by us previously (5).

Apoptotic cell death inducible by different doses of radiation will be determined by the semi-quantitative classical oligonucleosome-length DNA-fragmentation assay on agarose gels, flow analysis and subsequently quantitated by TUNEL. For TUNEL, cells will be seeded in chamber slides and 24 h later they will be exposed to various doses of radiation. The DNA will then be tailed with digoxigenin-dUTP and conjugated with anti-digoxigenin fluorescein. The specimen will be counterstained with propidium iodide and antifade and scored for apoptotic cells.

We will perform at least three experiments in each category to ascertain the reproducibility of the results. Data obtained on EGR-1 expression levels will be compared with the corresponding results on clonogenic survival, growth inhibition and apoptosis. Together, these experiments should provide information on the relationship between the radiation-induced kinetics of EGR-1 expression and the radiation response of prostate cancer cells (CWR22 and CWR22R) will be inferred in this specific aim.

Anticipated Results: It is expected that both, CWR22 and CWR22R cells, will be resistant to ionizing radiation than PC-3 cells irrespective of their *p53* status. It is also anticipated that CWR22R might be more radio-resistant than CWR22 cells.

Potential Problems and Alternate Methods: The only problem which we might foresee in this aim is the ability to form colonies by CWR22 and CWR22R cells for clonogenic-inhibition assay. To overcome this problem, we might use re-growth assays for each radiation dose. In this assay, by using trypan blue exclusion dye technique, we will generate growth curves for each graded radiation dose extended to about 120 hours after radiation. This approach will help us to obtain radiation survival information more appropriately.

Overlap with NIH grant: None

Specific aim #2: To determine the functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cell line CWR22R and the androgen-dependent CWR22. This will be performed in transfectant cell lines expressing the *Egr*-1 dominant-negative mutant or in parent cells where *Egr*-1 expression is blocked by *Egr*-1-antisense oligomers and also in transfectant cell lines overexpressing the cDNA of *Egr*-1.

Rationale : Radiation has been shown to induce a diverse spectrum of immediate-early genes. Selective suppression of a specific gene represents an important approach to understand the functional relevance of an immediate-early gene in the cascade leading to the ultimate phenotypic change. Suppression of gene expression can be achieved either by gene disruption or by introduction of a genetic element that inhibits the function of the target gene. Use of protein mutants that interfere with the function of the wild-type protein in a dominant fashion can be used to suppress the function of a specific gene (6). The antisense RNA approach involves the production of RNA sequences complementary to mRNA of the target gene (7). To study EGR-1 function, we will use a biologically active dominant negative mutant to suppress the function of the EGR-1 protein, or an antisense oligomer to suppress the expression of this gene. By using both these approaches to suppress the function or expression of *Egr*-1, we demonstrated that in melanoma cells EGR-1 is required for radiation-inducible tumor growth inhibition (5, 8). By contrast, we have previously shown that EGR-1 expression impedes the growth arrest action of interleukin-1 (8). Thus, in melanoma cell background, EGR-1 may play inducer-specific roles possibly by transregulating distinct sets of downstream target genes. As found recently in PC-3 cells, the dominant-negative mutant of EGR-1 confers radioresistance and the overexpression of *Egr*-1 cDNA enhances radiation sensitivity (9). This alteration of radiation response was demonstrated in two sets of PC-3 transfectant cell lines. In addition, our recent preliminary data suggest that EGR-1 protein regulates *p53*. We have shown, using genetically matched MEF cells, that radiation can down-regulate wild-type *p53* when *Egr*-1 function is absent (shown in rationale paragraph of specific aim 4). It will be of paramount interest to understand the radiation response profiles in CWR22 and CWR22R cells stably transfected with dominant-negative for *Egr*-1 or CMV-EGR-1 because these cell lines have varied *p53* levels (10). Thus, using a more formal approach we will determine whether the dominant negative mutant protects radiation-induced growth inhibition and the cDNA for *Egr*-1 enhances radiation-induced growth inhibition.

Experimental Design: We will confirm whether the dominant negative mutant can compete with co-transfected EGR-1 to transrepress expression of the EBS-CAT construct in CWR22 and CWR22R cells. The plasmid construct CMV-WT1-EGR1, CMV-EGR1 and vector alone (as control) will be transfected in CWR22 and CWR22R cells and stable transfectant cell lines will be selected with G418 sulfate. Two sets of transfectant cell lines will be irradiated at different doses (5, 10, or 20 Gy) and clonogenic survival and growth inhibition profiles will be determined by colony formation assay and [³H] thymidine-incorporation assays respectively. Basal levels of EGR-1 and radiation-inducible expression levels at different doses of radiation will be determined by immunohistochemistry and these expression levels will be quantitated by Western blot analysis. Apoptosis induced by different doses of radiation will be measured by DNA ladder test, flow analysis and TUNEL. In addition, to directly determine the specific function of EGR-1 in radiation-inducible growth inhibition and apoptosis, *Egr*-1 expression in the parental cells will be blocked with an antisense (AS) oligomer. Transient transfection with the nonsense (NS) oligomer will be used for control, as described previously (5). The irradiated cells will be subjected to [³H]thymidine incorporation experiments, immunocytochemistry and flow analysis & TUNEL, to determine the effect of the oligomers on radiation-inducible growth inhibition, EGR-1 expression, and apoptosis, respectively. At least three separate experiments using each of these procedures will be used to ascertain reproducibility of the results. Data obtained on cell survival, growth inhibition and apoptosis of two sets of transfected cells (having the plasmid contruct CMV-WT1-EGR1 and CMV-EGR1) will be compared to those corresponding to cell survival, growth inhibition and apoptosis of cells transfected with vector alone. Data obtained on growth inhibition and apoptosis of parental cells exposed to AS oligomer for EGR-1 will be compared individually to the data obtained on growth inhibition and apoptosis of cells exposed to either NS oligomer or no oligomer. These experiments should help elucidate the function of *Egr*-1 in radiation-inducible growth inhibition in prostate cancer cell lines.

4

Anticipated Results: Our recent data indicate that CWR22R cells contain modest level of EGR-1 protein expression (Figure 1, appendix A). Overexpressing *Egr*-1 cDNA or dominant-negative mutant in these cells may alter radiosensitivity which may relatively depend upon the p53 and TNF-α functional status.

Potential Problems and Alternate Methods: As stated earlier, the only potential problem in this aim will be on the ability to form colonies in CWR22 and CWR22R cells for clonogenic-inhibition assay. To overcome this problem, we will use re-growth assays to obtain radiation survival data.
Overlap with NIH grant: None

Specific aim #3: To elucidate the pathway of *Egr*-1 mediated regulation of p53 and TNF-α genes so that the differential mechanism of radiation-inducible apoptosis in prostate cancer cells (CWR22R) and the androgen-dependent CWR22 cells can be understood. This will be performed by studying the radiation-induced kinetics of p53 and TNF-α mRNA and protein in transfectant cell lines expressing the *Egr*-1 dominant negative mutant protein or in transfectant cell lines overexpressing the cDNA of *Egr*-1 by ^{32}P-RT-PCR and Western blot analysis respectively. Also, in these transfectants, CAT and gel-shift assays will be performed using the promoter of TNF-α and p53 to understand the transactivation mechanism by which EGR-1 may upregulate p53 and TNF-α.

Rationale: To identify potential downstream targets that might mediate the proapoptotic action of EGR-1, we conducted a GenBank search of genes that contain the EGR-1 consensus binding sites in their promoter regions, focusing on genes that satisfied the following stringent criteria : (i) they should be inducible by ionizing radiation; and (ii) they should be involved in apoptosis. One of the genes that met these criteria was tumor necrosis factor-α (TNF-α). The induction of TNF-α gene expression represents one aspect of cellular response to ionizing radiation that causes autocrine and paracrine tumor cell killing (11, 12 13). Ionizing radiation causes a transient increase in TNF-α mRNA followed by a corresponding increase in TNF-α protein (12). TNF-α protein is a well characterized cytokine that induces apoptosis in different cell types by binding to the TNF-R1 receptor. Binding to the TNF-R1 receptor triggers the sequential recruitment and activation of a cascade of death-domain containing proteins, that further activate cysteinyl-aspartate-specific proteinases (caspases) such as interleukin-converting enzyme and those of the interleukin-converting enzyme-related family (14, 15, 16-19). The caspases then cleave substrates such as poly (ADP-ribose) polymerase, nuclear lamins, actin, protein kinase C-δ and fodrin that are essential for cell survival leading to apoptotic cell death (14-17). An important example that may be relevant to radiation-induced growth control mechanism in many cell types is the regulation of TNF-α gene (20). A paracrine effect of TNF-α induction following x-rays was suggested since the media of irradiated cells were cytotoxic to other cell lines. Since the radiation-induced TNF-α has the property of killing the tumor cells (in addition this gene has a *Egr*-1 binding site in its promoter), there were few studies reported the effect of recombinant TNF-α on the growth of androgen-dependent and androgen-independent prostate tumor cells. In our recent report, we demonstrated that, in *p53*-deficient PC-3 cells, EGR-1 protein upregulates TNF-α by transactivation and binding to the EGR-1 consensus sequence present in the promoter of TNF-α gene. However, to ascertain the fact that this novel mechanism is ubiquitous in radiation treated advanced prostate tumors, we will perform experiments pertaining to the objectives of this specific aim which proposes to elucidate the mechanism by which EGR-1 may upregulate TNF-α and p53 in CWR22R (androgen-independent cell line) and CWR22 (androgen-dependent cell line).

One of the other gene which might play an important role in mediating the proapoptotic action of EGR-1 is *p53*, as documented by our preliminary study (shown in rationale paragraph of specific aim 4) and by previous report (21). The function of *p53* tumor suppressor protein is determined by various intrinsic properties of the protein. The effect of *p53* DNA-binding, and protein-protein interactions are determined by the conformation of protein. *p53* fulfils its role in cell cycle control and the onset of apoptotic cell death, which is altered when wild-type *p53* conformation changes due to a mutation. Furthermore, *p53* is involved in the expression of several growth factor and growth factor receptor genes. (22). Ionizing radiation is known to cause up-regulation of *p53* gene leading to G1 arrest (23). Thus, *p53* also fulfils the criteria that it is involved in mediating apoptosis, has EGR-1 binding sites in it's promoter region and is up-regulated by radiation. Our recent preliminary experiments demonstrated a novel mechanism involving the interaction of EGR-1 with *p53* in regulating apoptosis using genetically matched MEF cells with homozygous or heterozygous deletion of *Egr*-1 gene. However, to ascertain the fact that this novel mechanism is present in radiation treated advanced prostate tumors, we will perform experiments pertaining to the objectives of this specific aim which proposes to elucidate the mechanism by which ectopically or radiation-induced endogenous EGR-1 protein may upregulate *p53* in CWR22R and CWR22.

Experimental Design: We will first ascertained whether the recombinant TNF-α will cause growth inhibition and nucleosomal DNA fragmentation (apoptosis) in CWR22R and CWR22 cells. Growth inhibition will be performed by [³H] thymidine incorporation assay. Apoptosis by DNA ladder formation will be performed

5

by isolating genomic DNA from cells exposed to vehicle or recombinant TNF-α for 48 hours and this DNA will be subjected to 1.8% agarose gel electrophoresis. The kinetics of mRNA and protein levels of TNF-α will be intially determined in the untreated and irradiated parental cells CWR22R and CWR22 using ^{32}P-RT-PCR and ELISA respectively. Using two sets of transfectant cell lines obtained from each prostate tumor cell lines, the untreated and irradiated kinetics of mRNA and protein levels of TNF-α will be measured by ^{32}P-RT-PCR and ELISA respectively. For ^{32}P-RT-PCR analysis, total RNA extracts will be obtained at different intervals. The mRNA will be reverse transcribed to cDNA and PCR will be performed using specific radio-labeled primers for TNF-α. Using the products of the same reverse transcription, PCR will be performed using specific radio-labeled primers for internal control gene β-actin. Both the PCR products will be electrophoresed on 12% polyacrylamide gel. The bands will be read densitometrically and the β-actin levels will be normalised to obtain actual expression levels of TNF-α. The kinetic profile of parental cells will be compared with the transfectant group's TNF-α mRNA levels with their data on growth inhibition and apoptosis. This will help in understanding the role of TNF-α as a downstream mediator of *Egr*-1 function in radiation-inducible growth inhibition.

The kinetics of *p53* protein levels will be initially determined in the untreated and irradiated parental cells CWR22R and CWR22 using Western blot analysis. Using two sets of transfectant cell lines obtained from each prostate tumor cell line, the untreated and irradiated kinetics of *p53* protein levels will be measured by Western blot analysis. The kinetic profile of parental cells will be compared with the transfectant group's *p53* protein levels with their data on growth inhibition and apoptosis. This will help in understanding the role of *p53* as a downstream mediator of *Egr*-1 function in radiation-inducible growth inhibition.

To determine whether the EGR-1 protein in CWR22R and CWR22 cells can transactivate the TNF-α promoter, we will perform reporter assays using TNFp-CAT, TNFpmutant-CAT, p53(2.2+1.6)-CAT and p53(0.8)-CAT constructs. In our previous report, we documented that in PC-3 cells, an EGR-1 expression plasmid construct, CMV-EGR1 and radiation, has been shown to transactivate a CAT reporter plasmid construct, TNFp-CAT, which contains one EGR-1 binding sites present in the upstream of TNF-α promoter and CAT cDNA. Whereas, co-transfection with CMV-WT1-EGR-1 (dominant-negative mutant) or transient transfection with TNFpmutant-CAT (which contains mutation in the EGR-1 binding site) abrogated the reporter CAT activity (9). The constructs, p53(2.2+1.6)-CAT (121) and p53(0.8)-CAT (32), was kindly provided by Dr Rangnekar. Both constructs contain two EGR-1 binding sites. Using these constructs separately, CWR22R and CWR22 cells will be transiently transfected in the following manner : (a) TNFp-CAT / TNFpmutant-CAT / p53(2.2+1.6)-CAT / p53(0.8)-CAT alone; (b) TNFp-CAT / TNFpmutant-CAT / p53(2.2+1.6)-CAT / p53(0.8)-CAT+radiation; (c) TNFp-CAT / TNFpmutant-CAT / p53(2.2+1.6)-CAT / p53(0.8)-CAT+CMV-WT1-EGR-1; (d) TNFp-CAT / TNFpmutant-CAT / p53(2.2+1.6)-CAT / p53(0.8)-CAT+radiation+CMV-WT1-EGR-1; (e) TNFp-CAT / TNFpmutant-CAT / p53(2.2+1.6)-CAT / p53(0.8)-CAT + CMV-EGR1; (f) TNFp-CAT / TNFpmutant-CAT / p53(2.2+1.6)-CAT / p53(0.8)-CAT + CMV-EGR1+ radiation; (g) TNFp-CAT / TNFpmutant-CAT / p53(2.2+1.6)-CAT / p53(0.8)-CAT + CMV-EGR1+CMV-WT1-EGR-1; and (h) TNFp-CAT / TNFpmutant-CAT / p53(2.2+1.6)-CAT / p53(0.8)-CAT + CMV-EGR1+CMV-WT1-EGR-1+radiation. We will perform similar transient transfections, with modifications, using stable transfectants of CWR22R and CWR22 cells containing vector alone or CMV-EGR-1 or CMV-WT1-EGR-1. In addition, we will also perform electrophoretic mobility shift assay (gel-shifts) using short double-stranded DNA probe obtained from *p53* and TNF-α promoter containing either the *Egr*-1 binding sites. In brief, this will be performed by incubating the untreated or irradiated nuclear extracts obtained from parental and transfected CWR22R and CWR22 cells with ^{32}P-labeled probe. Then, the bound complexes will be separated from free probe by non-denaturing polyacrylamide gel electrophoresis. The gel will be run at 200 V for 2 hours, dried and autoradiographed. These experiments will help to understand the mechanism of transactivation by which radiation-induced EGR-1 protein upregulates *p53* and TNF-α.

Anticipated Results: Because of the direct binding sequence present in the promoters of *p53* and TNF-α, the ectopically expressed EGR-1 protein will transactivate *p53* and TNF-α promoters, whereas, CMV-WT1-EGR-1 will transrepress the *p53* and TNF-α promoters. This should occur in prostate cancer cell lines irrespective of their genetic make-up. However, activation of these CAT constructs by basal background and radiation-induced EGR-1 protein in these cells may entirely depend on the functional status of endogenous *Egr*-1 gene itself.

Potential Problems and Alternative Methods: As such, we do not foresee any potential problems in this specific aim.

Overlap with NIH grant: None

Specific aim #4: To understand the mechanism of radiation-induced apoptosis in isogenic mouse embryonic fibroblast (MEF) cells with homozygous (Egr-1$^{+/+}$), heterozygous (Egr-1$^{+/-}$) and null (Egr-1$^{-/-}$) allelic status for Egr-1 gene. This will be performed by studying the radiation-induced kinetics of Egr-1 target genes such as p53, TNF-α and pRB at the mRNA

6

and protein level in MEF cells with Egr-1$^{-/-}$ and Egr-1$^{+/+}$ background. In addition, CAT assays will be performed using p53-CAT, TNF-CAT and pRB-CAT constructs to understand the Egr-1 mediated regulation of transcription of p53, TNF-α and pRB genes.

Rationale: In our previous studies, we used tumor cell lines such as wild-type p53 melanoma cells (5) and p53-deficient prostate cancer cells (9) to understand the regulatory role of *Egr*-1 in apoptotic processes. These studies strongly suggested that *Egr*-1 can mediate its apoptotic action irrespective of *p53* status. However, in a recent report, it was found that *Egr*-1 transactivates the promoter of *p53* gene and up-regulates *p53* mRNA and protein levels in response to apoptotic stimuli (21). This prompted us to further investigate the interactive role of *Egr*-1 with *p53* during the process of apoptosis. We sought to investigate this mechanism in normal cell background with varied genomic status for *Egr*-1 gene (cells with both intact *Egr*-1 alleles, homozygous and heterozygous deletion for *Egr*-1 gene). Mouse embryonic fibroblast (MEF) cells established from homozygous (*Egr*-1$^{-/-}$) and heterozygous (*Egr*-1$^{+/-}$) *Egr*-1 knock-out mice were kindly provided by Dr Jeffrey Milbrandt, Dept of Pathology, Washington University, St.Louis (23). MEFs from normal mice containing intact alleles for *Egr*-1 was established in the laboratory. Using these cells, we demonstrate here that *Egr*-1 is the upstream regulator of apoptosis and this effect is mediated through the upregulation of *p53* protein. The preliminary study given below forms a supporting rationale for this specific aim to understand the mechanism of radiation-inducible apoptosis in normal cell background with Egr-1 gene as an upstream mediator.

Ionizing radiation causes enhanced cell death in *Egr*-1$^{+/-}$ cells. MEFs (*Egr*-1$^{-/-}$ and *Egr*-1$^{+/-}$ cells) were left untreated or irradiated at 5 Gy dose of ionizing radiation. TUNEL staining and flow cytometry was performed to determine the incidence of apoptosis. By TUNEL assay, the incidence of apoptosis after 24 hours of radiation was 3.5 % in *Egr*-1$^{-/-}$ cells and 22.8% in *Egr*-1$^{+/-}$ cells (Figure 2, appendix A). By flow cytometry assay using MC540 and Hoechst 342 staining, the incidence of apoptosis after 48 hours of radiation was 6.2 % in *Egr*-1$^{-/-}$ cells and 53% in *Egr*-1$^{+/-}$ cells (Figure 3 appendix A). Thus, ionizing radiation caused signifcantly enhanced apoptosis in *Egr*-1$^{+/-}$ cells (*p*<0.0001) when compared to *Egr*-1$^{-/-}$ cells as demonstrated by TUNEL and flow cytometry assays. These observations suggest that despite the presence of *p53* gene in this normal cell background, MEFs with homozygous deletion of *Egr*-1 were resistant to ionizing radiation-inducible apoptosis.

Ionizing radiation causes induction of EGR-1 protein in *Egr*-1$^{+/-}$ MEF cells. We examined whether EGR-1 induction was associated with enhanced apoptosis inducible by ionizing radiation in *Egr*-1$^{+/-}$ cells. Western blot analysis confirmed that *Egr*-1$^{+/-}$ and in *Egr*-1$^{-/-}$ cells no detectable basal levels of EGR-1 protein was found. Moreover, after exposure to a 5 Gy dose of radiation, *Egr*-1$^{+/-}$ cells showed induction of EGR-1 expression with peak levels (10 fold) at 30 min (Fig. 4 appendix A) and this was not evident in *Egr*-1$^{-/-}$ cells.

Egr-1$^{+/-}$ MEF cells exhibit EGR-1-dependent transcriptional activation via the GC-rich region in response to ionizing radiation. To ascertain the EGR-1-dependent transactivation process in *Egr*-1$^{-/-}$, *Egr*-1$^{+/-}$ and *Egr*-1$^{+/+}$ MEF cells, we performed transient transfections with (i) only reporter construct EBS-CAT that contains three tandem EGR-1-binding sites; (ii) EBS-CAT and an EGR-1 expression construct CMV-EGR-1; and (iii) EBS-CAT and ionizing radiation. As seen in Fig. 4, CAT activity was completely absent in basal and irradiated *Egr*-1$^{-/-}$ cells, whereas, CMV-EGR-1 elevated the CAT-activity. In *Egr*-1$^{+/-}$ and *Egr*-1$^{+/+}$ cells, ionizing radiation increased relative CAT activity in an allelic-dose dependent manner. A similar situation was observed in terms basal CAT activity in which *Egr*-1$^{+/+}$ cells showed slightly higher basal relative CAT levels when compared to *Egr*-1$^{+/-}$ cells. However, CMV-EGR-1 construct caused an increase in CAT reporter activity irrespective of endogenous *Egr*-1 allelic status (Figure 5 appendix A). These results confirmed that the EGR-1 protein is necessary for the transactivation of target genes containing EGR-1 binding sites.

Ionizing radiation causes down-regulation of *p53* protein in *Egr*-1$^{-/-}$ cells. The above observations have ascertained the fact that EGR-1 protein is necessary to cause radiation-induced apoptosis. Absence of EGR-1 protein renders enhanced resistance to radiation. Next, we sought to determine the role of *p53* in *Egr*-1$^{-/-}$ radioresistant cells. *Egr*-1$^{+/-}$ and *Egr*-1$^{-/-}$ cells were left untreated or irradiated at 5 Gy and proteins were extracted after each incubated time interval and subjected to Western blotting. *Egr*-1$^{+/-}$ cells showed a peak increase in *p53* levels to five fold in 3-6 hours. However, in *Egr*-1$^{-/-}$ cells, the *p53* protein was down-regulated in 1 hour after radiation and was completely absent in the later time points (Figure 6 appendix A). Thus, the loss of *p53* transactivation in *Egr*-1$^{-/-}$ cells might have contributed to enhanced resistance to apoptosis. The process of down-regulation and complete absence of *p53* protein in response to radiation remains unclear. However, it may speculated that mdm-2 may be up-regulated upon radiation damage and this protein may interact with nuclear *p53* protein to ubiquitously degrade the *p53* protein so that the cells can move from *p53*-mediated G$_1$ arrest to S-phase (25). Recently, it was reported that pRB regulates the stability of p53 via MDM-2 (26). Thus, degradation of p53 in irradiated *Egr*-1$^{-/-}$ MEF cells may relate to low levels of pRB protein present in *Egr*-1$^{-/-}$ MEFs. This observation underscores an important novel mechanism involving *Egr*-1 as a key upstream regulatory gene in the control of apoptosis process which is mediated through wild-type *p53* and pRB genes.

Experimental Design: This will be performed by analyzing mRNA and protein levels of p53, TNF-α and pRB using ^{32}P-RT-PCR and Western blot analysis. CAT assays will be performed in MEFs with different background for Egr-1 status using p53-, TNF- and pRB CAT constructs by transient transfections. In addition, immunoblot analysis will be performed to understand the dimeric complex (p53-mdm2) and trimeric complex (p53-mdm2-pRB) in untreated and irradiated cell lysates of MEFs with Egr-1$^{+/+}$ and Egr-1$^{-/-}$ background. These experiments will help to understand the mechanism of down-regulation of p53 protein in Egr-1$^{-/-}$ MEFs.

Anticipated results: Down-regulation of p53 protein in irradiated Egr-1-/- MEF may be due to reduced levels of pRB protein which regulates the p53 protein stability. Thus, for Egr-1 to mediate the apoptotic action of radiation, cells must contain functional p53 and pRB proteins.

Potential Problems and Alternative Methods: As such, we do not foresee any potential problems in this specific aim.

Overlap with NIH grant: None

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STATEMENT OF WORK

TASK-1. Radiobiological profile and *Egr*-1 gene characterization in CWR22R cells, months 1-4:
    a. Radiobiological characteristics of CWR22R and CWR22 cells (colony formation, growth inhibition and apoptosis) and radiation-induced *Egr*-1 protein analysis by Westerns and immunocytochemistry. Months 1-3.
    b. Characterization of *Egr*-1 gene in CWR22R and CWR22 cells (FISH, mutation analysis and sequencing of whole gene). Month 4.

TASK-2. Functional role of EGR-1 protein in the regulation of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22), months 9-14:
    a. Transfections using constructs such as empty vector (pCB6+), the chimera WT1-EGR1 and EGR-1 cDNA will be performed in CWR22R and CWR22 cells. CAT assays will be performed using EBS-CAT construct. Months 9-11.
    b. Radiobiological characteristics of stable transfectant (CWR22R and CWR22) cells (colony formation, growth inhibition and apoptosis). Months 12-13.
    c. Interpretation of results and preparation of manuscript for publication. Month 14.

TASK-3. Mechanism of radiation-inducible apoptosis in prostate cancer cells (CWR22R and CWR22) by *Egr*-1 mediated up-regulation of the TNF-α gene, months 15-18:
    a. CAT assays using p53 (2.2+1.6)-CAT, pRB-CAT and TNFp-CAT constructs in parental and stably transfected CWR22R and CWR22 cells. Gel-shift assays in parental and stably transfected CWR22R and CWR22 cells. Months 15-17.
    b. Interpretation of results and preparation of manuscript for publication. Month 18.

TASK-4. To understand the mechanism of radiation-induced apoptosis in isogenic mouse embryonic fibroblast (MEF) cells with homozygous (Egr-1$^{+/+}$), heterozygous (Egr-1$^{+/-}$) and null (Egr-1$^{-/-}$) allelic status for Egr-1 gene. Months 5-8.
    a. Western blot and RT-PCR analysis of p53, TNF-a and pRB genes in untreated and irradiated MEFs with Egr-1+/+ and Egr-1-/- background. Month 5.
    b. CAT assays for p53, TNF-a and pRB. Month 6.
    c. Immunblot analysis of p53, mdm-2 and pRB. Month 7
    c. Interpretation of results and preparation of manuscript for publication. Month 8.